

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 October 2002 (10.10.2002)

PCT

(10) International Publication Number
WO 02/079444 A2

- (51) International Patent Classification⁷: C12N (74) Agents: BURKE, John, E. et al.; Deltagen, Inc., 740 Bay Road, Redwood City, CA 94063 (US).
- (21) International Application Number: PCT/US02/09854
- (22) International Filing Date: 29 March 2002 (29.03.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/280,370 29 March 2001 (29.03.2001) US
10/112,286 28 March 2002 (28.03.2002) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:
US 60/280,370 (CON)
Filed on 29 March 2001 (29.03.2001)
US 10/112,286 (CON)
Filed on 28 March 2002 (28.03.2002)
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): DELTAGEN, INC. [US/US]; 740 Bay Road, Redwood City, CA 94063 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): ALLEN, Keith, D. [US/US]; 224 Custer Trail, Cary, NC 27513 (US).
- Published:
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: TRANSGENIC MICE CONTAINING MSK2 SERINE/THREONINE KINASE GENE DISRUPTIONS

(57) Abstract: The present invention relates to transgenic animals, as well as compositions and methods relating to the characterization of gene function. Specifically, the present invention provides transgenic mice comprising mutations in a MSK2 gene. Such transgenic mice are useful as models for disease and for identifying agents that modulate gene expression and gene function, and as potential treatments for various disease states and disease conditions.

WO 02/079444 A2

TRANSGENIC MICE CONTAINING MSK2 SERINE/THREONINE KINASE GENE DISRUPTIONS

Field of the Invention

5 The present invention relates to transgenic animals, compositions and methods relating to the characterization of gene function.

Background of the Invention

Protein kinases are a large family of proteins that share a homologous catalytic domain of 250 to 300 amino acids, which can be subdivided into 11 subdomains. Protein kinases frequently play key roles in the normal regulation of growth and development in eukaryotic organisms. As a
10 consequence, aberrant expression or mutations in this family of molecules frequently result in transformation.

One important subfamily of kinases is the serine/threonine kinases (or STKs). One serine/threonine kinase is the mitogen- and stress-activated protein kinase-2 (MSK2). The complete 3120 bp mRNA cds for murine MSK2 has been deposited in GenBank (Accession number:
15 AF074714; GI: 3786405).

Given the importance of kinases, particularly STKs such as MSK2, a clear need exists for identification and characterization of kinases which can play a role in preventing, ameliorating or correcting dysfunctions or diseases.

Summary of the Invention

20 The present invention generally relates to transgenic animals, as well as to compositions and methods relating to the characterization of gene function.

The present invention provides transgenic cells comprising a disruption in a MSK2 gene. The transgenic cells of the present invention are comprised of any cells capable of undergoing homologous recombination. Preferably, the cells of the present invention are stem cells and more
25 preferably, embryonic stem (ES) cells, and most preferably, murine ES cells. According to one embodiment, the transgenic cells are produced by introducing a targeting construct into a stem cell to produce a homologous recombinant, resulting in a mutation of the MSK2 gene. In another embodiment, the transgenic cells are derived from the transgenic animals described below. The cells derived from the transgenic animals includes cells that are isolated or present in a tissue or organ, and
30 any cell lines or any progeny thereof.

The present invention also provides a targeting construct and methods of producing the targeting construct that when introduced into stem cells produces a homologous recombinant. In one embodiment, the targeting construct of the present invention comprises first and second polynucleotide sequences that are homologous to the MSK2 gene. The targeting construct may also comprise a
35 polynucleotide sequence that encodes a selectable marker that is preferably positioned between the

two different homologous polynucleotide sequences in the construct. The targeting construct may also comprise other regulatory elements that can enhance homologous recombination.

The present invention further provides non-human transgenic animals and methods of producing such non-human transgenic animals comprising a disruption in a MSK2 gene. The transgenic animals of the present invention include transgenic animals that are heterozygous and homozygous for a null mutation in the MSK2 gene. In one aspect, the transgenic animals of the present invention are defective in the function of the MSK2 gene. In another aspect, the transgenic animals of the present invention comprise a phenotype associated with having a mutation in a MSK2 gene. Preferably, the transgenic animals are rodents and, most preferably, are mice.

In a preferred embodiment, the present invention provides a transgenic mouse comprising a disruption in a MSK2 gene, wherein there is no native expression of the endogenous MSK2 gene.

In accordance with one aspect of the present invention, transgenic mice having a disruption in the MSK2 gene exhibit a growth abnormality. The transgenic mice exhibiting a growth abnormality show one or more of the following: decreased body weight, decreased body length, decreased body weight to body weight ratio, thin appearance or decreased organ weights. The decrease in organ weights may be observed in spleen, liver, thymus, kidney, heart or testes. The transgenic mice of the present invention may alternatively or additionally exhibit a histopathological abnormality, which may be one or more of: a small liver nodule or carcinoma, especially carcinomas of the tongue.

In one aspect of the present invention, a transgenic mouse having a disruption in the MSK2 gene exhibits a phenotype consistent with one or more symptoms of a disease associated with MSK2.

In one aspect of the present invention, a transgenic mouse having a disruption in the MSK2 gene exhibits an increased startle response in a startle test. This result is consistent with one or more symptoms of anxiety.

The present invention also provides methods of identifying agents capable of affecting a phenotype of a transgenic animal. For example, a putative agent is administered to the transgenic animal and a response of the transgenic animal to the putative agent is measured and compared to the response of a "normal" or wild-type mouse, or alternatively compared to a transgenic animal control (without agent administration). The invention further provides agents identified according to such methods. The present invention also provides methods of identifying agents useful as therapeutic agents for treating conditions associated with a disruption or other mutation (including naturally occurring mutations) of the MSK2 gene.

One aspect of the present invention relates to a method of identifying a potential therapeutic agent for the treatment of a disease associated with the MSK2 gene, in which the method includes the steps of: administering the potential therapeutic agent to a transgenic mouse having a disruption in a MSK2 gene; and determining whether the potential therapeutic agent modulates the disease associated

with the MSK2 gene, wherein the modulation of the disease identifies a potential therapeutic agent for the treatment of that disease.

One aspect of the present invention relates to a method of identifying a potential therapeutic agent for the treatment of anxiety, in which the method includes the steps of: administering the potential therapeutic agent to a transgenic mouse having a disruption in a MSK2 gene; and determining
5 whether the potential therapeutic agent modulates a symptom of consistent with that seen in anxiety patients, wherein the modulation of the symptom identifies a potential therapeutic agent for the treatment of anxiety.

A further aspect of the present invention provides a method of identifying a potential
10 therapeutic agent for the treatment of a disease associated with the MSK2 gene, in which the method includes the steps of: contacting the potential therapeutic agent with MSK2 gene product; and determining whether the potential therapeutic agent modulates that product, wherein modulation of the gene product identifies a potential therapeutic agent for the treatment of the disease associated with the MSK2 gene.

15 A further aspect of the present invention provides a method of identifying a potential therapeutic agent for the treatment of anxiety, in which the method includes the steps of: contacting the potential therapeutic agent with MSK2 gene product; and determining whether the potential therapeutic agent modulates that product, wherein modulation of the gene product identifies a potential therapeutic agent for the treatment of anxiety.

20 The present invention further provides a method of identifying agents having an effect on MSK2 expression or function. The method includes administering an effective amount of the agent to a transgenic animal, preferably a mouse. The method includes measuring a response of the transgenic animal, for example, to the agent, and comparing the response of the transgenic animal to a control animal, which may be, for example, a wild-type animal or alternatively, a transgenic animal control.
25 Compounds that may have an effect on MSK2 expression or function may also be screened against cells in cell-based assays, for example, to identify such compounds.

The invention also provides cell lines comprising nucleic acid sequences of a MSK2 gene. Such cell lines may be capable of expressing such sequences by virtue of operable linkage to a promoter functional in the cell line. Preferably, expression of the MSK2 gene sequence is under the
30 control of an inducible promoter. Also provided are methods of identifying agents that interact with the MSK2 gene, comprising the steps of contacting the MSK2 gene with an agent and detecting an agent/MSK2 gene complex. Such complexes can be detected by, for example, measuring expression of an operably linked detectable marker.

The invention further provides methods of treating diseases or conditions associated with a
35 disruption in a MSK2 gene, and more particularly, to a disruption or other alteration in the expression or function of the MSK2 gene. In a preferred embodiment, methods of the present invention involve

treating diseases or conditions associated with a disruption or other alteration in the MSK2 gene's expression or function, including administering to a subject in need, a therapeutic agent that affects MSK2 expression or function. In accordance with this embodiment, the method comprises administration of a therapeutically effective amount of a natural, synthetic, semi-synthetic, or recombinant MSK2 gene, MSK2 gene products or fragments thereof as well as natural, synthetic, semi-synthetic or recombinant analogs.

In one aspect of the present invention, a therapeutic agent for treating a disease associated with the MSK2 gene modulates the MSK2 gene product. Another aspect of the present invention relates to a therapeutic agent for treating a disease associated with the MSK2 gene, in which the agent is an agonist or antagonist of the MSK2 gene product.

In one aspect of the present invention, a therapeutic agent for treating anxiety modulates the MSK2 gene product. Another aspect of the present invention relates to a therapeutic agent for treating anxiety, in which the agent is an agonist of the MSK2 gene product.

The present invention also provides compositions comprising or derived from ligands or other molecules or compounds that bind to or interact with MSK2, including agonists or antagonists of MSK2. Such agonists or antagonists of MSK2 include antibodies and antibody mimetics, as well as other molecules that can readily be identified by routine assays and experiments well known in the art.

The present invention further provides methods of treating diseases or conditions associated with disrupted targeted gene expression or function, wherein the methods comprise detecting and replacing through gene therapy mutated or otherwise defective or abnormal MSK2 genes.

Definitions

The term "gene" refers to (a) a gene containing at least one of the DNA sequences disclosed herein; (b) any DNA sequence that encodes the amino acid sequence encoded by the DNA sequences disclosed herein and/or; (c) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein. Preferably, the term includes coding as well as noncoding regions, and preferably includes all sequences necessary for normal gene expression including promoters, enhancers and other regulatory sequences.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes single-, double-stranded and triple helical molecules.

"Oligonucleotide" refers to polynucleotides of between 5 and about 100 nucleotides of single- or double-stranded DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes, or chemically synthesized by methods known in the art. A "primer" refers to an oligonucleotide, usually single-stranded, that provides a 3'-hydroxyl end for the initiation of enzyme-

mediated nucleic acid synthesis. The following are non-limiting embodiments of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A nucleic acid molecule may also comprise
5 modified nucleic acid molecules, such as methylated nucleic acid molecules and nucleic acid molecule analogs. Analogs of purines and pyrimidines are known in the art, and include, but are not limited to, aziridinycytosine, 4-acetylcytosine, 5-fluorouracil, 5-bromouracil, 5-carboxymethylamino-methyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-
10 methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, pseudouracil, 5-pentynyl-uracil and 2,6-diaminopurine. The use of uracil as a substitute for thymine in a deoxyribonucleic acid is also considered an analogous form of pyrimidine.

A "fragment" of a polynucleotide is a polynucleotide comprised of at least 9 contiguous nucleotides, preferably at least 15 contiguous nucleotides and more preferably at least 45 nucleotides,
15 of coding or non-coding sequences.

The term "gene targeting" refers to a type of homologous recombination that occurs when a fragment of genomic DNA is introduced into a mammalian cell and that fragment locates and recombines with endogenous homologous sequences.

The term "homologous recombination" refers to the exchange of DNA fragments between
20 two DNA molecules or chromatids at the site of homologous nucleotide sequences.

The term "homologous" as used herein denotes a characteristic of a DNA sequence having at least about 70 percent sequence identity as compared to a reference sequence, typically at least about 85 percent sequence identity, preferably at least about 95 percent sequence identity, and more preferably about 98 percent sequence identity, and most preferably about 100 percent sequence
25 identity as compared to a reference sequence. Homology can be determined using, for example, a "BLASTN" algorithm. It is understood that homologous sequences can accommodate insertions, deletions and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking
30 sequence, or a repetitive portion of a chromosome.

The term "target gene" (alternatively referred to as "target gene sequence" or "target DNA sequence" or "target sequence") refers to any nucleic acid molecule, polynucleotide, or gene to be modified by homologous recombination. The target sequence includes an intact gene, an exon or intron, a regulatory sequence or any region between genes. The target gene may comprise a portion
35 of a particular gene or genetic locus in the individual's genomic DNA. As provided herein, the target gene of the present invention is a MSK2 gene, or a homolog or ortholog thereof. A "MSK2 gene"

refers to a sequence comprising SEQ ID NO:1 or comprising the MSK2 sequence identified in GenBank as Accession No.: AF074714; GI: 3786405, or orthologs or homologs thereof.

“Disruption” of a MSK2 gene occurs when a fragment of genomic DNA locates and recombines with an endogenous homologous sequence. These sequence disruptions or modifications may include insertions, missense, frameshift, deletion, or substitutions, or replacements of DNA sequence, or any combination thereof. Insertions include the insertion of entire genes, which may be of animal, plant, fungal, insect, prokaryotic, or viral origin. Disruption, for example, can alter or replace a promoter, enhancer, or splice site of a MSK2 gene, and can alter the normal gene product by inhibiting its production partially or completely or by enhancing the normal gene product’s activity. In a preferred embodiment, the disruption is a null disruption, wherein there is no significant expression of the MSK2 gene.

The term “native expression” refers to the expression of the full-length polypeptide encoded by the MSK2 gene, at expression levels present in the wild-type mouse. Thus, a disruption in which there is “no native expression” of the endogenous MSK2 gene refers to a partial or complete reduction of the expression of at least a portion of a polypeptide encoded by an endogenous MSK2 gene of a single cell, selected cells, or all of the cells of a mammal. The term “knockout” is a synonym for functional inactivation of the gene.

The term “construct” or “targeting construct” refers to an artificially assembled DNA segment to be transferred into a target tissue, cell line or animal. Typically, the targeting construct will include a gene or a nucleic acid sequence of particular interest, a marker gene and appropriate control sequences. As provided herein, the targeting construct of the present invention comprises a MSK2 targeting construct. A “MSK2 targeting construct” includes a DNA sequence homologous to at least one portion of a MSK2 gene and is capable of producing a disruption in a MSK2 gene in a host cell.

The term “transgenic cell” refers to a cell containing within its genome a MSK2 gene that has been disrupted, modified, altered, or replaced completely or partially by the method of gene targeting.

The term “transgenic animal” refers to an animal that contains within its genome a specific gene that has been disrupted or otherwise modified or mutated by the method of gene targeting. “Transgenic animal” includes both the heterozygous animal (*i.e.*, one defective allele and one wild-type allele) and the homozygous animal (*i.e.*, two defective alleles).

As used herein, the terms “selectable marker” and “positive selection marker” refer to a gene encoding a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (*Neo*^r) gene are resistant to the compound G418. Cells that do not carry the *Neo*^r gene marker are killed by G418. Other positive selection markers are known to, or are within the purview of, those of ordinary skill in the art.

A "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) or for incorporation of nucleic acid molecules and/or proteins. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent due to natural, accidental, or deliberate mutation. A host cell includes cells transfected with the constructs of the present invention.

The term "modulates" or "modulation" as used herein refers to the decrease, inhibition, reduction, amelioration, increase or enhancement of a MSK2 function, expression, activity, or alternatively a phenotype associated with a disruption in a MSK2 gene. The term "ameliorates" or "amelioration" as used herein refers to a decrease, reduction or elimination of a condition, disease, disorder, or phenotype, including an abnormality or symptom associated with a disruption in a MSK2 gene.

The term "abnormality" refers to any disease, disorder, condition, or phenotype in which a disruption of a MSK2 gene is implicated, including pathological conditions and behavioral observations.

Brief Description of the Drawings

Figure 1 shows the polynucleotide sequence for a mouse MSK2 gene (SEQ ID NO:1).

Figure 2 shows the amino acid sequence for murine MSK2 (SEQ ID NO:2).

Figures 3-4 show the location and extent of the disrupted portion of the MSK2 gene, as well as the nucleotide sequences flanking the *Neo*^r insert in the targeting construct. Figure 4 shows the sequences identified as SEQ ID NO:3 and SEQ ID NO:4, which were used in the 5'- and 3'- targeting arms (including the homologous sequences) of the MSK2 targeting construct, respectively.

Detailed Description of the Invention

The invention is based, in part, on the evaluation of the expression and role of genes and gene expression products, primarily those associated with a MSK2 gene. Among other uses or applications, the invention permits the definition of disease pathways and the identification of diagnostically and therapeutically useful targets. For example, genes that are mutated or down-regulated under disease conditions may be involved in causing or exacerbating the disease condition. Treatments directed at up-regulating the activity of such genes or treatments that involve alternate pathways, may ameliorate the disease condition.

Generation of Targeting Construct

The targeting construct of the present invention may be produced using standard methods known in the art. (*see, e.g.*, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; E.N. Glover (eds.), 1985, *DNA Cloning: A Practical Approach*, Volumes I and II; M.J. Gait (ed.), 1984, *Oligonucleotide Synthesis*; B.D. Hames & S.J. Higgins (eds.), 1985, *Nucleic Acid Hybridization*; B.D. Hames & S.J. Higgins (eds.), 1984, *Transcription and Translation*; R.I. Freshney (ed.), 1986, *Animal*

Cell Culture; Immobilized Cells and Enzymes, IRL Press, 1986; B. Perbal, 1984, A Practical Guide To Molecular Cloning; F.M. Ausubel *et al.*, 1994, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). For example, the targeting construct may be prepared in accordance with conventional ways, where sequences may be synthesized, isolated from natural sources, manipulated, cloned, ligated, subjected to *in vitro* mutagenesis, primer repair, or the like. At various stages, the joined sequences may be cloned, and analyzed by restriction analysis, sequencing, or the like.

The targeting DNA can be constructed using techniques well known in the art. For example, the targeting DNA may be produced by chemical synthesis of oligonucleotides, nick-translation of a double-stranded DNA template, polymerase chain-reaction amplification of a sequence (or ligase chain reaction amplification), purification of prokaryotic or target cloning vectors harboring a sequence of interest (*e.g.*, a cloned cDNA or genomic DNA, synthetic DNA or from any of the aforementioned combination) such as plasmids, phagemids, YACs, cosmids, bacteriophage DNA, other viral DNA or replication intermediates, or purified restriction fragments thereof, as well as other sources of single and double-stranded polynucleotides having a desired nucleotide sequence. Moreover, the length of homology may be selected using known methods in the art. For example, selection may be based on the sequence composition and complexity of the predetermined endogenous target DNA sequence(s).

The targeting construct of the present invention typically comprises a first sequence homologous to a portion or region of the MSK2 gene and a second sequence homologous to a second portion or region of the MSK2 gene. The targeting construct may further comprise a positive selection marker, which is preferably positioned in between the first and the second DNA sequences that are homologous to a portion or region of the target DNA sequence. The positive selection marker may be operatively linked to a promoter and a polyadenylation signal.

Other regulatory sequences known in the art may be incorporated into the targeting construct to disrupt or control expression of a particular gene in a specific cell type. In addition, the targeting construct may also include a sequence coding for a screening marker, for example, green fluorescent protein (GFP), or another modified fluorescent protein.

Although the size of the homologous sequence is not critical and can range from as few as about 15-20 base pairs to as many as 100 kb, preferably each fragment is greater than about 1 kb in length, more preferably between about 1 and about 10 kb, and even more preferably between about 1 and about 5 kb. One of skill in the art will recognize that although larger fragments may increase the number of homologous recombination events in ES cells, larger fragments will also be more difficult to clone.

In a preferred embodiment of the present invention, the targeting construct is prepared directly from a plasmid genomic library using the methods described in pending U.S. Patent Application Ser. No.: 08/971,310, filed November 17, 1997, the disclosure of which is incorporated

herein in its entirety. Generally, a sequence of interest is identified and isolated from a plasmid library in a single step using, for example, long-range PCR. Following isolation of this sequence, a second polynucleotide that will disrupt the target sequence can be readily inserted between two regions encoding the sequence of interest. In accordance with this aspect, the construct is generated in two steps by (1) amplifying (for example, using long-range PCR) sequences homologous to the target sequence, and (2) inserting another polynucleotide (for example a selectable marker) into the PCR product so that it is flanked by the homologous sequences. Typically, the vector is a plasmid from a plasmid genomic library. The completed construct is also typically a circular plasmid.

In another embodiment, the targeting construct is designed in accordance with the regulated positive selection method described in U.S. Patent Application Ser. No. 09/954,483, filed September 17, 2001, the disclosure of which is incorporated herein in its entirety. The targeting construct is designed to include a PGK-*neo* fusion gene having two *lacO* sites, positioned in the PGK promoter and an NLS-*lacI* gene comprising a lac repressor fused to sequences encoding the NLS from the SV40 T antigen.

In another embodiment, the targeting construct may contain more than one selectable marker gene, including a negative selectable marker, such as the herpes simplex virus tk (HSV-tk) gene. The negative selectable marker may be operatively linked to a promoter and a polyadenylation signal. (see, e.g., U.S. Patent No. 5,464,764; U.S. Patent No. 5,487,992; U.S. Patent No. 5,627,059; and U.S. Patent No. 5,631,153).

Generation of Cells and Confirmation of Homologous Recombination Events

Once an appropriate targeting construct has been prepared, the targeting construct may be introduced into an appropriate host cell using any method known in the art. Various techniques may be employed in the present invention, including, for example: pronuclear microinjection; retrovirus mediated gene transfer into germ lines; gene targeting in embryonic stem cells; electroporation of embryos; sperm-mediated gene transfer; and calcium phosphate/DNA co-precipitates, microinjection of DNA into the nucleus, bacterial protoplast fusion with intact cells, transfection, polycations, e.g., polybrene, polyornithine, etc., or the like (see, e.g., U.S. Patent No. 4,873,191; Van der Putten *et al.*, 1985, *Proc. Natl. Acad. Sci., USA* 82:6148-6152; Thompson *et al.*, 1989, *Cell* 56:313-321; Lo, 1983, *Mol Cell. Biol.* 3:1803-1814; Lavitrano *et al.*, 1989, *Cell*, 57:717-723). Various techniques for transforming mammalian cells are known in the art. (see, e.g., Gordon, 1989, *Intl. Rev. Cytol.*, 115:171-229; Keown *et al.*, 1989, *Methods in Enzymology*; Keown *et al.*, 1990, *Methods and Enzymology*, Vol. 185, pp. 527-537; Mansour *et al.*, 1988, *Nature*, 336:348-352).

In a preferred aspect of the present invention, the targeting construct is introduced into host cells by electroporation. In this process, electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the construct. The pores created during

electroporation permit the uptake of macromolecules such as DNA. (*see, e.g.*, Potter, H. et al., 1984, *Proc. Nat'l. Acad. Sci. U.S.A.* 81:7161-7165).

Any cell type capable of homologous recombination may be used in the practice of the present invention. Examples of such target cells include cells derived from vertebrates including mammals such as humans, bovine species, ovine species, murine species, simian species, and other eucaryotic organisms such as filamentous fungi, and higher multicellular organisms such as plants.

Preferred cell types include embryonic stem (ES) cells, which are typically obtained from pre-implantation embryos cultured *in vitro*. (*see, e.g.*, Evans, M. J. et al., 1981, *Nature* 292:154-156; Bradley, M. O. et al., 1984, *Nature* 309:255-258; Gossler et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:9065-9069; and Robertson et al., 1986, *Nature* 322:445-448). The ES cells are cultured and prepared for introduction of the targeting construct using methods well known to the skilled artisan. (*see, e.g.*, Robertson, E. J. ed. "Teratocarcinomas and Embryonic Stem Cells, a Practical Approach", IRL Press, Washington D.C., 1987; Bradley et al., 1986, *Current Topics in Devel. Biol.* 20:357-371; by Hogan et al., in "Manipulating the Mouse Embryo": A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y., 1986; Thomas et al., 1987, *Cell* 51:503; Koller et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88:10730; Dorin et al., 1992, *Transgenic Res.* 1:101; and Veis et al., 1993, *Cell* 75:229). The ES cells that will be inserted with the targeting construct are derived from an embryo or blastocyst of the same species as the developing embryo into which they are to be introduced. ES cells are typically selected for their ability to integrate into the inner cell mass and contribute to the germ line of an individual when introduced into the mammal in an embryo at the blastocyst stage of development. Thus, any ES cell line having this capability is suitable for use in the practice of the present invention.

The present invention may also be used to knock out or otherwise modify or disrupt genes in other cell types, such as stem cells. By way of example, stem cells may be myeloid, lymphoid, or neural progenitor and precursor cells. These cells comprising a knock out, modification or disruption of a gene may be particularly useful in the study of MSK2 gene function in individual developmental pathways. Stem cells may be derived from any vertebrate species, such as mouse, rat, dog, cat, pig, rabbit, human, non-human primates and the like.

After the targeting construct has been introduced into cells, the cells in which successful gene targeting has occurred are identified. Insertion of the targeting construct into the targeted gene is typically detected by identifying cells for expression of the marker gene. In a preferred embodiment, the cells transformed with the targeting construct of the present invention are subjected to treatment with an appropriate agent that selects against cells not expressing the selectable marker. Only those cells expressing the selectable marker gene survive and/or grow under certain conditions. For example, cells that express the introduced neomycin resistance gene are resistant to the compound G418, while cells that do not express the neo gene marker are killed by G418. If the targeting

construct also comprises a screening marker such as GFP, homologous recombination can be identified through screening cell colonies under a fluorescent light. Cells that have undergone homologous recombination will have deleted the GFP gene and will not fluoresce.

5 If a regulated positive selection method is used in identifying homologous recombination events, the targeting construct is designed so that the expression of the selectable marker gene is regulated in a manner such that expression is inhibited following random integration but is permitted (derepressed) following homologous recombination. More particularly, the transfected cells are screened for expression of the *neo* gene, which requires that (1) the cell was successfully electroporated, and (2) *lac* repressor inhibition of *neo* transcription was relieved by homologous
10 recombination. This method allows for the identification of transfected cells and homologous recombinants to occur in one step with the addition of a single drug.

Alternatively, a positive-negative selection technique may be used to select homologous recombinants. This technique involves a process in which a first drug is added to the cell population, for example, a neomycin-like drug to select for growth of transfected cells, *i.e.* positive selection. A
15 second drug, such as FIAU is subsequently added to kill cells that express the negative selection marker, *i.e.* negative selection. Cells that contain and express the negative selection marker are killed by a selecting agent, whereas cells that do not contain and express the negative selection marker survive. For example, cells with non-homologous insertion of the construct express HSV thymidine kinase and therefore are sensitive to the herpes drugs such as gancyclovir (GANC) or FIAU (1-(2-
20 deoxy 2-fluoro-B-D-arabinofluranosyl)-5-iodouracil). (*see, e.g., Mansour et al., Nature* 336:348-352: (1988); Capecchi, *Science* 244:1288-1292, (1989); Capecchi, *Trends in Genet.* 5:70-76 (1989)).

Successful recombination may be identified by analyzing the DNA of the selected cells to confirm homologous recombination. Various techniques known in the art, such as PCR and/or Southern analysis may be used to confirm homologous recombination events.

25 Homologous recombination may also be used to disrupt genes in stem cells, and other cell types, which are not totipotent embryonic stem cells. By way of example, stem cells may be myeloid, lymphoid, or neural progenitor and precursor cells. Such transgenic cells may be particularly useful in the study of MSK2 gene function in individual developmental pathways. Stem cells may be derived from any vertebrate species, such as mouse, rat, dog, cat, pig, rabbit, human, non-human
30 primates and the like.

In cells that are not totipotent, it may be desirable to knock out both copies of the target using methods that are known in the art. For example, cells comprising homologous recombination at a target locus that have been selected for expression of a positive selection marker (*e.g., Neo'*) and screened for non-random integration, can be further selected for multiple copies of the selectable
35 marker gene by exposure to elevated levels of the selective agent (*e.g., G418*). The cells are then analyzed for homozygosity at the target locus. Alternatively, a second construct can be generated

with a different positive selection marker inserted between the two homologous sequences. The two constructs can be introduced into the cell either sequentially or simultaneously, followed by appropriate selection for each of the positive marker genes. The final cell is screened for homologous recombination of both alleles of the target.

5 Production of Transgenic Animals

Selected cells are then injected into a blastocyst (or other stage of development suitable for the purposes of creating a viable animal, such as, for example, a morula) of an animal (*e.g.*, a mouse) to form chimeras (*see e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed., IRL, Oxford, pp. 113-152 (1987)). Alternatively, selected ES cells
10 can be allowed to aggregate with dissociated mouse embryo cells to form the aggregation chimera. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Chimeric progeny harbouring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA. In one embodiment, chimeric progeny mice are used to generate a mouse with a
15 heterozygous disruption in the MSK2 gene. Heterozygous transgenic mice can then be mated. It is well known in the art that typically $\frac{1}{4}$ of the offspring of such matings will have a homozygous disruption in the MSK2 gene.

The heterozygous and homozygous transgenic mice can then be compared to normal, wild-type mice to determine whether disruption of the MSK2 gene causes phenotypic changes, especially
20 pathological changes. For example, heterozygous and homozygous mice may be evaluated for phenotypic changes by physical examination, necropsy, histology, clinical chemistry, complete blood count, body weight, organ weights, and cytological evaluation of bone marrow. Phenotypic changes may also comprise behavioral modifications or abnormalities.

In one embodiment, the phenotype (or phenotypic change) associated with a disruption in the
25 MSK2 gene is placed into or stored in a database. Preferably, the database includes: (i) genotypic data (*e.g.*, identification of the disrupted gene) and (ii) phenotypic data (*e.g.*, phenotype(s) resulting from the gene disruption) associated with the genotypic data. The database is preferably electronic. In addition, the database is preferably combined with a search tool so that the database is searchable.

Conditional Transgenic Animals

30 The present invention further contemplates conditional transgenic or knockout animals, such as those produced using recombination methods. Bacteriophage P1 Cre recombinase and flp recombinase from yeast plasmids are two non-limiting examples of site-specific DNA recombinase enzymes that cleave DNA at specific target sites (lox P sites for cre recombinase and frt sites for flp recombinase) and catalyze a ligation of this DNA to a second cleaved site. A large number of suitable
35 alternative site-specific recombinases have been described, and their genes can be used in accordance

with the method of the present invention. Such recombinases include the Int recombinase of bacteriophage λ (with or without Xis) (Weisberg, R. *et al.*, in *Lambda II*, (Hendrix, R. *et al.*, Eds.), Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 211-50 (1983), herein incorporated by reference); TpnI and the β -lactamase transposons (Mercier *et al.*, *J. Bacteriol.*, 172:3745-57 (1990));

5 the Tn3 resolvase (Flanagan & Fennwald *J. Molec. Biol.*, 206:295-304 (1989); Stark *et al.*, *Cell*, 58:779-90 (1989)); the yeast recombinases (Matsuzaki *et al.*, *J. Bacteriol.*, 172:610-18 (1990)); the *B. subtilis* SpoIVC recombinase (Sato *et al.*, *J. Bacteriol.* 172:1092-98 (1990)); the Flp recombinase (Schwartz & Sadowski, *J. Molec. Biol.*, 205:647-658 (1989); Parsons *et al.*, *J. Biol. Chem.*, 265:4527-33 (1990); Golic & Lindquist, *Cell*, 59:499-509 (1989); Amin *et al.*, *J. Molec. Biol.*,

10 214:55-72 (1990)); the Hin recombinase (Glasgow *et al.*, *J. Biol. Chem.*, 264:10072-82 (1989)); immunoglobulin recombinases (Malynn *et al.*, *Cell*, 54:453-460 (1988)); and the Cin recombinase (Haffter & Bickle, *EMBO J.*, 7:3991-3996 (1988); Hubner *et al.*, *J. Molec. Biol.*, 205:493-500 (1989)), all herein incorporated by reference. Such systems are discussed by Echols (*J. Biol. Chem.* 265:14697-14700 (1990)); de Villartay (*Nature*, 335:170-74 (1988)); Craig, (*Ann. Rev. Genet.*,

15 22:77-105 (1988)); Poyart-Salmeron *et al.*, (*EMBO J.* 8:2425-33 (1989)); Hunger-Bertling *et al.*, (*Mol. Cell. Biochem.*, 92:107-16 (1990)); and Cregg & Madden (*Mol. Gen. Genet.*, 219:320-23 (1989)), all herein incorporated by reference.

Cre has been purified to homogeneity, and its reaction with the loxP site has been extensively characterized (Abremski & Hess *J. Mol. Biol.* 259:1509-14 (1984), herein incorporated by reference).

20 Cre protein has a molecular weight of 35,000 and can be obtained commercially from New England Nuclear/Du Pont. The cre gene (which encodes the Cre protein) has been cloned and expressed (Abremski *et al.*, *Cell* 32:1301-11 (1983), herein incorporated by reference). The Cre protein mediates recombination between two loxP sequences (Sternberg *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 45:297-309 (1981)), which may be present on the same or different DNA molecule.

25 Because the internal spacer sequence of the loxP site is asymmetrical, two loxP sites can exhibit directionality relative to one another (Hoess & Abremski *Proc. Natl. Acad. Sci. U.S.A.* 81:1026-29 (1984)). Thus, when two sites on the same DNA molecule are in a directly repeated orientation, Cre will excise the DNA between the sites (Abremski *et al.*, *Cell* 32:1301-11 (1983)). However, if the sites are inverted with respect to each other, the DNA between them is not excised after

30 recombination but is simply inverted. Thus, a circular DNA molecule having two loxP sites in direct orientation will recombine to produce two smaller circles, whereas circular molecules having two loxP sites in an inverted orientation simply invert the DNA sequences flanked by the loxP sites. In addition, recombinase action can result in reciprocal exchange of regions distal to the target site when targets are present on separate DNA molecules.

35 Recombinases have important application for characterizing gene function in knockout models. When the constructs described herein are used to disrupt MSK2 genes, a fusion transcript

can be produced when insertion of the positive selection marker occurs downstream (3') of the translation initiation site of the MSK2 gene. The fusion transcript could result in some level of protein expression with unknown consequence. It has been suggested that insertion of a positive selection marker gene can affect the expression of nearby genes. These effects may make it difficult to determine gene function after a knockout event since one could not discern whether a given phenotype is associated with the inactivation of a gene, or the transcription of nearby genes. Both potential problems are solved by exploiting recombinase activity. When the positive selection marker is flanked by recombinase sites in the same orientation, the addition of the corresponding recombinase will result in the removal of the positive selection marker. In this way, effects caused by the positive selection marker or expression of fusion transcripts are avoided.

In one embodiment, purified recombinase enzyme is provided to the cell by direct microinjection. In another embodiment, recombinase is expressed from a co-transfected construct or vector in which the recombinase gene is operably linked to a functional promoter. An additional aspect of this embodiment is the use of tissue-specific or inducible recombinase constructs that allow the choice of when and where recombination occurs. One method for practicing the inducible forms of recombinase-mediated recombination involves the use of vectors that use inducible or tissue-specific promoters or other gene regulatory elements to express the desired recombinase activity. The inducible expression elements are preferably operatively positioned to allow the inducible control or activation of expression of the desired recombinase activity. Examples of such inducible promoters or other gene regulatory elements include, but are not limited to, tetracycline, metallothionine, ecdysone, and other steroid-responsive promoters, rapamycin responsive promoters, and the like (No *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:3346-51 (1996); Furth *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:9302-6 (1994)). Additional control elements that can be used include promoters requiring specific transcription factors such as viral, promoters. Vectors incorporating such promoters would only express recombinase activity in cells that express the necessary transcription factors.

Models for Disease

The cell- and animal-based systems described herein can be utilized as models for diseases. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate disease animal models. In addition, cells from humans may be used. These systems may be used in a variety of applications. Such assays may be utilized as part of screening strategies designed to identify agents, such as compounds that are capable of ameliorating disease symptoms. Thus, the animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions that may be effective in treating disease.

Cell-based systems may be used to identify compounds that may act to ameliorate disease symptoms. For example, such cell systems may be exposed to a compound suspected of exhibiting an

ability to ameliorate disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed cells. After exposure, the cells are examined to determine whether one or more of the disease cellular phenotypes has been altered to resemble a more normal or more wild-type, non-disease phenotype.

5 In addition, animal-based disease systems, such as those described herein, may be used to identify compounds capable of ameliorating disease symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies, and interventions that may be effective in treating a disease or other phenotypic characteristic of the animal. For example, animal models may be exposed to a compound or agent suspected of exhibiting an ability to ameliorate
10 disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with the disease. Exposure may involve treating mother animals during gestation of the model animals described herein, thereby exposing embryos or fetuses to the compound or agent that may prevent or ameliorate the disease or phenotype.
15 Neonatal, juvenile, and adult animals can also be exposed.

 More particularly, using the animal models of the invention, methods of identifying agents are provided, in which such agents can be identified on the basis of their ability to affect at least one phenotype associated with a disruption in a MSK2 gene. In one embodiment, the present invention provides a method of identifying agents having an effect on MSK2 expression or function. The
20 method includes measuring a physiological response of the animal, for example, to the agent and comparing the physiological response of such animal to a control animal, wherein the physiological response of the animal comprising a disruption in a MSK2 as compared to the control animal indicates the specificity of the agent. A "physiological response" is any biological or physical parameter of an animal that can be measured. Molecular assays (*e.g.*, gene transcription, protein
25 production and degradation rates), physical parameters (*e.g.*, exercise physiology tests, measurement of various parameters of respiration, measurement of heart rate or blood pressure and measurement of bleeding time), behavioral testing, and cellular assays (*e.g.*, immunohistochemical assays of cell surface markers, or the ability of cells to aggregate or proliferate) can be used to assess a physiological response.

30 The transgenic animals and cells of the present invention may be utilized as models for diseases, disorders, or conditions associated with phenotypes relating to a disruption in a MSK2 gene.

 The present invention provides a unique animal model for testing and developing new treatments relating to the behavioral phenotypes. Analysis of the behavioral phenotype allows for the development of an animal model useful for testing, for instance, the efficacy of proposed genetic and
35 pharmacological therapies for human genetic diseases, such as neurological, neuropsychological, or psychotic illnesses.

A statistical analysis of the various behaviors measured can be carried out using any conventional statistical program routinely used by those skilled in the art (such as, for example, "Analysis of Variance" or ANOVA). A "p" value of about 0.05 or less is generally considered to be statistically significant, although slightly higher p values may still be indicative of statistically significant differences. To statistically analyze abnormal behavior, a comparison is made between the behavior of a transgenic animal (or a group thereof) to the behavior of a wild-type mouse (or a group thereof), typically under certain prescribed conditions. "Abnormal behavior" as used herein refers to behavior exhibited by an animal having a disruption in the MSK2 gene, *e.g.* transgenic animal, which differs from an animal without a disruption in the MSK2 gene, *e.g.* wild-type mouse. Abnormal behavior consists of any number of standard behaviors that can be objectively measured (or observed) and compared. In the case of comparison, it is preferred that the change be statistically significant to confirm that there is indeed a meaningful behavioral difference between the knockout animal and the wild-type control animal. Examples of behaviors that may be measured or observed include, but are not limited to, ataxia, rapid limb movement, eye movement, breathing, motor activity, cognition, emotional behaviors, social behaviors, hyperactivity, hypersensitivity, anxiety, impaired learning, abnormal reward behavior, and abnormal social interaction, such as aggression.

A series of tests may be used to measure the behavioral phenotype of the animal models of the present invention, including neurological and neuropsychological tests to identify abnormal behavior. These tests may be used to measure abnormal behavior relating to, for example, learning and memory, eating, pain, aggression, sexual reproduction, anxiety, depression, schizophrenia, and drug abuse. (*see, e.g.,* Crawley & Paylor, *Hormones and Behavior* 31:197-211 (1997)).

The social interaction test involves exposing a mouse to other animals in a variety of settings. The social behaviors of the animals (*e.g.,* touching, climbing, sniffing, and mating) are subsequently evaluated. Differences in behaviors can then be statistically analyzed and compared (*see, e.g.,* S. E. File et al., *Pharmacol. Bioch. Behav.* 22:941-944 (1985); R. R. Holson, *Phys. Behav.* 37:239-247 (1986)). Exemplary behavioral tests include the following.

The mouse startle response test typically involves exposing the animal to a sensory (typically auditory) stimulus and measuring the startle response of the animal (*see, e.g.,* M. A. Geyer et al., *Brain Res. Bull.* 25:485-498 (1990); Paylor and Crawley, *Psychopharmacology* 132:169-180 (1997)).

The startle test screens for changes in the basic fundamental nervous system or muscle-related functions. This includes changes in 1) hearing - auditory processing; 2) sensory and motor processing - related to the auditory circuit and culminating in a motor related output; 3) motor abnormalities, including skeletal muscle or motor neuron related changes; and 4) anxiety levels. The startle reflex is a short-latency response of the skeletal musculature elicited by a sudden auditory stimulus. The startle reflex is seen across many species, making the startle response test a useful animal model for studying abnormalities in the neural control of simple behaviors and searching for

treatments and causes of those abnormalities. In rats or mice, the response is usually measured in a response chamber, which allows the measurement of the whole-body flinch elicited by the stimulus. Similar stimuli are used to test the response in humans, where a blink response is measured using electromyography of the orbicularis oculi muscle.

5 One component of the startle reflex test is prepulse inhibition (PPI). PPI is the reduction or gating of the startle reflex response produced by a weak prestimulus presented at a brief interval, usually between 30-500ms, before the startle eliciting stimulus. Both rats and humans have been exhibit a graded increase in PPI with increasing prepulse intensities.

10 Deficits in PPI are observed in human schizophrenic patients. Deficits in PPI have been associated with dopamine overactivity, as shown by the ability to produce a loss of PPI in rats treated with dopamine agonists, such as apomorphine. PPI can be restored in apomorphine treated rats by antipsychotics in a manner that correlates with clinical antipsychotic potency and D₂ receptor affinity. It is also believed that neural modulation of PPI in rats is affected by circuitry linking the hippocampus (HPC), the nucleus accumbens (NAC), the subpallidum, and the pontine reticular formation.

15 Aside from dopaminergic involvement in PPI and sensory gating, both forebrain glutamatergic and serotonergic systems have been implicated in the pathophysiology of schizophrenia and the action of atypical antipsychotics, and both glutamatergic and serotonergic activity are important substrates modulating PPI in rats. Non competitive NMDA glutamate receptor antagonists and serotonin receptor (particularly 5-HT_{1B}) agonists have both been shown to reduce PPI in rats.

20 Genetic factors may be critical determinants of sensorimotor gating in rats. This has been supported by studies showing strain related differences in the dopaminergic modulation of PPI, as well as the production through inbreeding of strains of rats whose behavior was either apomorphine-sensitive or insensitive. Rats having a disruption of the 5-HT_{1B} were reported to have slightly elevated basal PPI compared to wild-type controls, indicating a tonic regulation of PPI by 5-HT_{1B}.

25 This conclusion was supported by research showing that a 5-HT_{1A/1B} agonist reduced PPI in wild-type mice, but not in the 5-HT_{1B} knockouts. The investigation of the effects on PPI of disruptions of other genes could be a valuable tool for understanding the role of particular gene products in the regulation of PPI and sensorimotor gating.

30 The connection between the abnormalities in sensorimotor gating in schizophrenic patients and PPI are supported by the belief that brain regions frequently implicated in the pathophysiology of the disorder are also involved in the regulation of PPI. Abnormalities at several levels of the startle gating circuitry, including the hippocampus, nucleus accumbens, striatum, globus pallidus, and thalamus, have been noted in schizophrenic patients.

35 The electric shock test generally involves exposure to an electrified surface and measurement of subsequent behaviors such as, for example, motor activity, learning, social behaviors. The behaviors are measured and statistically analyzed using standard statistical tests. (*see, e.g., G. J. Kant*

et al., *Pharm. Bioch. Behav.* 20:793-797 (1984); N. J. Leidenheimer et al., *Pharmacol. Bioch. Behav.* 30:351-355 (1988)).

The tail-pinch or immobilization test involves applying pressure to the tail of the animal and/or restraining the animal's movements. Motor activity, social behavior, and cognitive behavior are examples of the areas that are measured. (see, e.g., M. Bertolucci D'Angic et al., *Neurochem.* 55:1208-1214 (1990)).

The novelty test generally comprises exposure to a novel environment and/or novel objects. The animal's motor behavior in the novel environment and/or around the novel object are measured and statistically analyzed. (see, e.g., D. K. Reinstein et al., *Pharm. Bioch. Behav.* 17:193-202 (1982); B. Poucet, *Behav. Neurosci.* 103:1009-10016 (1989); R. R. Holson et al., *Phys. Behav.* 37:231-238 (1986)). This test may be used to detect visual processing deficiencies or defects.

The learned helplessness test involves exposure to stresses, for example, noxious stimuli, which cannot be affected by the animal's behavior. The animal's behavior can be statistically analyzed using various standard statistical tests. (see, e.g., A. Leshner et al., *Behav. Neural Biol.* 26:497-501 (1979)).

Alternatively, a tail suspension test may be used, in which the "immobile" time of the mouse is measured when suspended "upside-down" by its tail. This is a measure of whether the animal struggles, an indicator of depression. In humans, depression is believed to result from feelings of a lack of control over one's life or situation. It is believed that a depressive state can be elicited in animals by repeatedly subjecting them to aversive situations over which they have no control. A condition of "learned helplessness" is eventually reached, in which the animal will stop trying to change its circumstances and simply accept its fate. Animals that stop struggling sooner are believed to be more prone to depression. Studies have shown that the administration of certain antidepressant drugs prior to testing increases the amount of time that animals struggle before giving up.

The Morris water-maze test comprises learning spatial orientations in water and subsequently measuring the animal's behaviors, such as, for example, by counting the number of incorrect choices. The behaviors measured are statistically analyzed using standard statistical tests. (see, e.g., E. M. Spruijt et al., *Brain Res.* 527:192-197 (1990)).

Alternatively, a Y-shaped maze may be used (see, e.g., McFarland, D.J., *Pharmacology, Biochemistry and Behavior* 32:723-726 (1989); Dellu, F. et al., *Neurobiology of Learning and Memory* 73:31-48 (2000)). The Y-maze is generally believed to be a test of cognitive ability. The dimensions of each arm of the Y-maze can be, for example, approximately 40 cm x 8 cm x 20 cm, although other dimensions may be used. Each arm can also have, for example, sixteen equally spaced photobeams to automatically detect movement within the arms. At least two different tests can be performed using such a Y-maze. In a continuous Y-maze paradigm, mice are allowed to explore all three arms of a Y-maze for, e.g., approximately 10 minutes. The animals are continuously tracked

using photobeam detection grids, and the data can be used to measure spontaneous alteration and positive bias behavior. Spontaneous alteration refers to the natural tendency of a "normal" animal to visit the least familiar arm of a maze. An alternation is scored when the animal makes two consecutive turns in the same direction, thus representing a sequence of visits to the least recently entered arm of the maze. Position bias determines egocentrically defined responses by measuring the animal's tendency to favor turning in one direction over another. Therefore, the test can detect differences in an animal's ability to navigate on the basis of allocentric or egocentric mechanisms. The two-trial Y-maze memory test measures response to novelty and spatial memory based on a free-choice exploration paradigm. During the first trial (acquisition), the animals are allowed to freely visit two arms of the Y-maze for, *e.g.*, approximately 15 minutes. The third arm is blocked off during this trial. The second trial (retrieval) is performed after an intertrial interval of, *e.g.*, approximately 2 hours. During the retrieval trial, the blocked arm is opened and the animal is allowed access to all three arms for, *e.g.*, approximately 5 minutes. Data are collected during the retrieval trial and analyzed for the number and duration of visits to each arm. Because the three arms of the maze are virtually identical, discrimination between novelty and familiarity is dependent on "environmental" spatial cues around the room relative to the position of each arm. Changes in arm entry and duration of time spent in the novel arm in a transgenic animal model may be indicative of a role of that gene in mediating novelty and recognition processes.

The passive avoidance or shuttle box test generally involves exposure to two or more environments, one of which is noxious, providing a choice to be learned by the animal. Behavioral measures include, for example, response latency, number of correct responses, and consistency of response. (*see, e.g.*, R. Ader et al., *Psychon. Sci.* 26:125-128 (1972); R. R. Holson, *Phys. Behav.* 37:221-230 (1986)). Alternatively, a zero-maze can be used. In a zero-maze, the animals can, for example, be placed in a closed quadrant of an elevated annular platform having, *e.g.*, 2 open and 2 closed quadrants, and are allowed to explore for approximately 5 minutes. This paradigm exploits an approach-avoidance conflict between normal exploratory activity and an aversion to open spaces in rodents. This test measures anxiety levels and can be used to evaluate the effectiveness of anti-anxiolytic drugs. The time spent in open quadrants versus closed quadrants may be recorded automatically, with, for example, the placement of photobeams at each transition site.

The food avoidance test involves exposure to novel food and objectively measuring, for example, food intake and intake latency. The behaviors measured are statistically analyzed using standard statistical tests. (*see, e.g.*, B. A. Campbell et al., *J. Comp. Physiol. Psychol.* 67:15-22 (1969)).

The elevated plus-maze test comprises exposure to a maze, without sides, on a platform, the animal's behavior is objectively measured by counting the number of maze entries and maze learning.

The behavior is statistically analyzed using standard statistical tests. (*see, e.g.,* H. A. Baldwin et al., *Brain Res. Bull.*, 20:603-606 (1988)).

The stimulant-induced hyperactivity test involves injection of stimulant drugs (*e.g.,* amphetamines, cocaine, PCP, and the like), and objectively measuring, for example, motor activity, social interactions, cognitive behavior. The animal's behaviors are statistically analyzed using standard statistical tests. (*see, e.g.,* P. B. S. Clarke et al., *Psychopharmacology* 96:511-520 (1988); P. Kuczenski et al., *J. Neuroscience* 11:2703-2712 (1991)).

The self-stimulation test generally comprises providing the mouse with the opportunity to regulate electrical and/or chemical stimuli to its own brain. Behavior is measured by frequency and pattern of self-stimulation. Such behaviors are statistically analyzed using standard statistical tests. (*see, e.g.,* S. Nassif et al., *Brain Res.*, 332:247-257 (1985); W. L. Isaac et al., *Behav. Neurosci.* 103:345-355 (1989)).

The reward test involves shaping a variety of behaviors, *e.g.,* motor, cognitive, and social, measuring, for example, rapidity and reliability of behavioral change, and statistically analyzing the behaviors measured. (*see, e.g.,* L. E. Jarrard et al., *Exp. Brain Res.* 61:519-530 (1986)).

The DRL (differential reinforcement to low rates of responding) performance test involves exposure to intermittent reward paradigms and measuring the number of proper responses, *e.g.,* lever pressing. Such behavior is statistically analyzed using standard statistical tests. (*see, e.g.,* J. D. Sinden et al., *Behav. Neurosci.* 100:320-329 (1986); V. Nalwa et al., *Behav Brain Res.* 17:73-76 (1985); and A. J. Nonneman et al., *J. Comp. Physiol. Psych.* 95:588-602 (1981)).

The spatial learning test involves exposure to a complex novel environment, measuring the rapidity and extent of spatial learning, and statistically analyzing the behaviors measured. (*see, e.g.,* N. Pitsikas et al., *Pharm. Bioch. Behav.* 38:931-934 (1991); B. Poucet et al., *Brain Res.* 37:269-280 (1990); D. Christie et al., *Brain Res.* 37:263-268 (1990); and F. Van Haaren et al., *Behav. Neurosci.* 102:481-488 (1988)). Alternatively, an open-field (of) test may be used, in which the greater distance traveled for a given amount of time is a measure of the activity level and anxiety of the animal. When the open field is a novel environment, it is believed that an approach-avoidance situation is created, in which the animal is "torn" between the drive to explore and the drive to protect itself. Because the chamber is lighted and has no places to hide other than the corners, it is expected that a "normal" mouse will spend more time in the corners and around the periphery than it will in the center where there is no place to hide. "Normal" mice will, however, venture into the central regions as they explore more and more of the chamber. It can then be extrapolated that especially anxious mice will spend most of their time in the corners, with relatively little or no exploration of the central region, whereas bold (*i.e.,* less anxious) mice will travel a greater distance, showing little preference for the periphery versus the central region.

The visual, somatosensory and auditory neglect tests generally comprise exposure to a sensory stimulus, objectively measuring, for example, orientating responses, and statistically analyzing the behaviors measured. (*see, e.g.,* J. M. Vargo et al., *Exp. Neurol.* 102:199-209 (1988)).

5 The consummatory behavior test generally comprises feeding and drinking, and objectively measuring quantity of consumption. The behavior measured is statistically analyzed using standard statistical tests. (*see, e.g.,* P. J. Fletcher et al., *Psychopharmacol.* 102:301-308 (1990); M. G. Corda et al., *Proc. Nat'l Acad. Sci. USA* 80:2072-2076 (1983)).

A visual discrimination test can also be used to evaluate the visual processing of an animal. One or two similar objects are placed in an open field and the animal is allowed to explore for about 10 5-10 minutes. The time spent exploring each object (proximity to, *i.e.,* movement within, *e.g.,* about 3-5 cm of the object is considered exploration of an object) is recorded. The animal is then removed from the open field, and the objects are replaced by a similar object and a novel object. The animal is returned to the open field and the percent time spent exploring the novel object over the old object is measured (again, over about a 5-10 minute span). "Normal" animals will typically spend a higher 15 percentage of time exploring the novel object rather than the old object. If a delay is imposed between sampling and testing, the memory task becomes more hippocampal-dependent. If no delay is imposed, the task is more based on simple visual discrimination. This test can also be used for olfactory discrimination, in which the objects (preferably, simple blocks) can be sprayed or otherwise treated to hold an odor. This test can also be used to determine if the animal can make gustatory 20 discriminations; animals that return to the previously eaten food instead of novel food exhibit gustatory neophobia.

A hot plate analgesia test can be used to evaluate an animal's sensitivity to heat or painful stimuli. For example, a mouse can be placed on an approximately 55°C hot plate and the mouse's response latency (*e.g.,* time to pick up and lick a hind paw) can be recorded. These responses are not 25 reflexes, but rather "higher" responses requiring cortical involvement. This test may be used to evaluate a nociceptive disorder.

A tail-flick test may also be used to evaluate an animal's sensitivity to heat or painful stimuli. For example, a high-intensity thermal stimulus can be directed to the tail of a mouse and the mouse's response latency recorded (*e.g.,* the time from onset of stimulation to a rapid flick/withdrawal from 30 the heat source) can be recorded. These responses are simple nociceptive reflexive responses that are involuntary spinally mediated flexion reflexes. This test may also be used to evaluate a nociceptive disorder.

An accelerating rotarod test may be used to measure coordination and balance in mice. Animals can be, for example, placed on a rod that acts like a rotating treadmill (or rolling log). The 35 rotarod can be made to rotate slowly at first and then progressively faster until it reaches a speed of, *e.g.,* approximately 60 rpm. The mice must continually reposition themselves in order to avoid falling

off. The animals are preferably tested in at least three trials, a minimum of 20 minutes apart. Those mice that are able to stay on the rod the longest are believed to have better coordination and balance.

A metrazol administration test can be used to screen animals for varying susceptibilities to seizures or similar events. For example, a 5mg/ml solution of metrazol can be infused through the tail vein of a mouse at a rate of, *e.g.*, approximately 0.375 ml/min. The infusion will cause all mice to experience seizures, followed by death. Those mice that enter the seizure stage the soonest are believed to be more prone to seizures. Four distinct physiological stages can be recorded: soon after the start of infusion, the mice will exhibit a noticeable "twitch", followed by a series of seizures, ending in a final tensing of the body known as "tonic extension", which is followed by death.

10 MSK2 Gene Products

The present invention further contemplates use of the MSK2 gene sequence to produce MSK2 gene products. MSK2 gene products may include proteins that represent functionally equivalent gene products. Such an equivalent gene product may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the gene sequences described herein, but which result in a silent change, thus producing a functionally equivalent MSK2 gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Functionally equivalent", as utilized herein, refers to a protein capable of exhibiting a substantially similar *in vivo* activity as the endogenous gene products encoded by the MSK2 gene sequences. Alternatively, when utilized as part of an assay, "functionally equivalent" may refer to peptides capable of interacting with other cellular or extracellular molecules in a manner substantially similar to the way in which the corresponding portion of the endogenous gene product would.

Other protein products useful according to the methods of the invention are peptides derived from or based on the MSK2 gene products produced by recombinant or synthetic means (derived peptides).

MSK2 gene products may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the gene polypeptides and peptides of the invention by expressing nucleic acids encoding gene sequences are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing gene protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo*

recombination/genetic recombination (*see, e.g.*, Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*). Alternatively, RNA capable of encoding gene protein sequences may be chemically synthesized using, for example, automated synthesizers (*see, e.g.* Oligonucleotide Synthesis: A Practical Approach, Gait, M. J. ed., IRL Press, Oxford (1984)).

5 A variety of host-expression vector systems may be utilized to express the gene coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the gene protein of the invention *in situ*. These include but are not limited to microorganisms such as bacteria
10 (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing gene protein coding sequences; yeast (*e.g.* *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the gene protein coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the gene protein coding sequences; plant cell systems infected with recombinant virus
15 expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing gene protein coding sequences; or mammalian cell systems (*e.g.* COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter;
20 the vaccinia virus 7.5 K promoter).

 In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the gene protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, vectors that direct the expression of high levels of fusion protein products that
25 are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, *EMBO J.*, 2:1791-94 (1983)), in which the gene protein coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.*, 13:3101-09 (1985); Van Heeke *et al.*, *J. Biol. Chem.*, 264:5503-9 (1989)); and the like. pGEX vectors may also be
30 used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned MSK2 gene protein can be released from the GST moiety.

35 In a preferred embodiment, full length cDNA sequences are appended with in-frame Bam HI sites at the amino terminus and Eco RI sites at the carboxyl terminus using standard PCR

methodologies (Innis et al. (eds) PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego (1990)) and ligated into the pGEX-2TK vector (Pharmacia, Uppsala, Sweden). The resulting cDNA construct contains a kinase recognition site at the amino terminus for radioactive labeling and glutathione S-transferase sequences at the carboxyl terminus for affinity purification
5 (Nilsson et al., *EMBO J.*, 4: 1075-80 (1985); Zabeau et al., *EMBO J.*, 1: 1217-24 (1982)).

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).
10 Successful insertion of gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (*see, e.g.*, Smith et al., *J. Virol.* 46: 584-93 (1983); U.S. Patent No. 4,745,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the gene coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1
20 or E3) will result in a recombinant virus that is viable and capable of expressing gene protein in infected hosts. (*e.g.*, *see* Logan et al., *Proc. Natl. Acad. Sci. USA*, 81:3655-59 (1984)). Specific initiation signals may also be required for efficient translation of inserted gene coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate
25 expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the gene coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of
30 origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (*see* Bitter et al., *Methods in Enzymol.*, 153:516-44 (1987)).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such
35 modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific

mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the gene protein may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells that stably integrate the plasmid into their chromosomes and grow, to form foci, which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the gene protein.

In a preferred embodiment, timing and/or quantity of expression of the recombinant protein can be controlled using an inducible expression construct. Inducible constructs and systems for inducible expression of recombinant proteins will be well known to those skilled in the art. Examples of such inducible promoters or other gene regulatory elements include, but are not limited to, tetracycline, metallothionine, ecdysone, and other steroid-responsive promoters, rapamycin responsive promoters, and the like (No et al., *Proc. Natl. Acad. Sci. USA*, 93:3346-51 (1996); Furth et al., *Proc. Natl. Acad. Sci. USA*, 91:9302-6 (1994)). Additional control elements that can be used include promoters requiring specific transcription factors such as viral, particularly HIV, promoters. In one embodiment, a Tet inducible gene expression system is utilized. (Gossen et al., *Proc. Natl. Acad. Sci. USA*, 89:5547-51 (1992); Gossen et al., *Science*, 268:1766-69 (1995)). Tet Expression Systems are based on two regulatory elements derived from the tetracycline-resistance operon of the *E. coli* Tn10 transposon—the tetracycline repressor protein (TetR) and the tetracycline operator sequence (*tetO*) to which TetR binds. Using such a system, expression of the recombinant protein is placed under the control of the *tetO* operator sequence and transfected or transformed into a host cell. In the presence of TetR, which is co-transfected into the host cell, expression of the recombinant protein is repressed due to binding of the TetR protein to the *tetO* regulatory element. High-level, regulated gene expression can then be induced in response to varying concentrations of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox), which compete with *tetO* elements for binding to

TetR. Constructs and materials for tet inducible gene expression are available commercially from CLONTECH Laboratories, Inc., Palo Alto, CA.

When used as a component in an assay system, the gene protein may be labeled, either directly or indirectly, to facilitate detection of a complex formed between the gene protein and a test substance. Any of a variety of suitable labeling systems may be used including but not limited to radioisotopes such as ^{125}I ; enzyme labeling systems that generate a detectable calorimetric signal or light when exposed to substrate; and fluorescent labels. Where recombinant DNA technology is used to produce the gene protein for such assay systems, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection.

Indirect labeling involves the use of a protein, such as a labeled antibody, which specifically binds to the gene product. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library.

Production of Antibodies

Described herein are methods for the production of antibodies capable of specifically recognizing one or more epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')_2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a MSK2 gene in a biological sample, or, alternatively, as a method for the inhibition of abnormal MSK2 gene activity. Thus, such antibodies may be utilized as part of disease treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of MSK2 gene proteins, or for the presence of abnormal forms of such proteins.

For the production of antibodies, various host animals may be immunized by injection with the MSK2 gene, its expression product or a portion thereof. Such host animals may include but are not limited to rabbits, mice, rats, goats and chickens, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as MSK2 gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with gene product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Köhler and Milstein, *Nature*, 256:495-7 (1975); and U.S. Patent No. 4,376,110, the human B-cell hybridoma technique (Kosbor et al., *Immunology Today*, 4:72 (1983); Cote et al., *Proc. Natl. Acad. Sci. USA*, 80:2026-30 (1983)), and the EBV-hybridoma technique (Cole et al., in *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., New York, pp. 77-96 (1985)). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci.*, 81:6851-6855 (1984); Takeda et al., *Nature*, 314:452-54 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778; Bird, *Science* 242:423-26 (1988); Huston et al., *Proc. Natl. Acad. Sci. USA*, 85:5879-83 (1988); and Ward et al., *Nature*, 334:544-46 (1989)) can be adapted to produce gene-single chain antibodies. Single chain antibodies are typically formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., *Science*, 246:1275-81 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Screening Methods

Various animal-derived "preparations," including cells and tissues, as well as cell-free extracts, homogenates, fractions and purified proteins, may be used to determine whether a particular agent is capable of modulating an activity of a MSK2 serine/threonine kinase or a phenotype associated therewith. For example, such preparations may be generated according to methods well known in the art from the tissues or organs of wild-type and knockout animals. Wild-type, but not knockout, preparations will contain endogenous MSK2 serine/threonine kinase, as well as the native activities, interactions and effects of the MSK2 serine/threonine kinase. Thus, when knockout and wild-type preparations are contacted with a test agent in parallel, the ability of the test agent to

modulate MSK2 serine/threonine kinases, or a phenotype associated therewith, can be determined. Agents capable of modulating an activity of a MSK2 serine/threonine kinase or a phenotype associated therewith are identified as those that modulate wild-type, but not knockout, preparations. Modulation may be detected, for example, as the ability of the agent to interact with a preparation, thereby indicating interaction with the gene product itself or a product thereof. Alternatively, the agent may affect a structural, metabolic or biochemical feature of the preparation, such as enzymatic activity of the preparation related to the MSK2 serine/threonine kinase. An inclusive discussion of the events for which modulation by a test agent may be observed is beyond the scope of this application, but will be well known by those skilled in the art.

The present invention may be employed in a process for screening for agents such as agonists, *i.e.*, agents that bind to and activate MSK2 polypeptides, or antagonists, *i.e.*, inhibit the activity or interaction of MSK2 polypeptides with its ligand. Thus, polypeptides of the invention may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures as known in the art. Any methods routinely used to identify and screen for agents that can modulate receptors may be used in accordance with the present invention.

The present invention provides methods for identifying and screening for agents that modulate MSK2 expression or function. More particularly, cells that contain and express MSK2 gene sequences may be used to screen for therapeutic agents. Such cells may include non-recombinant monocyte cell lines, such as U937 (ATCC# CRL-1593), THP-1 (ATCC# TIB-202), and P388D1 (ATCC# TIB-63); endothelial cells such as HUVEC's and bovine aortic endothelial cells (BAEC's); as well as generic mammalian cell lines such as HeLa cells and COS cells, *e.g.*, COS-7 (ATCC# CRL-1651). Further, such cells may include recombinant, transgenic cell lines. For example, the transgenic mice of the invention may be used to generate cell lines, containing one or more cell types involved in a disease, that can be used as cell culture models for that disorder. While cells, tissues, and primary cultures derived from the disease transgenic animals of the invention may be utilized, the generation of continuous cell lines is preferred. For examples of techniques that may be used to derive a continuous cell line from the transgenic animals, see Small et al., *Mol. Cell Biol.*, 5:642-48 (1985).

MSK2 gene sequences may be introduced into and overexpressed in, the genome of the cell of interest. In order to overexpress a MSK2 gene sequence, the coding portion of the MSK2 gene sequence may be ligated to a regulatory sequence that is capable of driving gene expression in the cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilized in the absence of undue experimentation. MSK2 gene sequences may also be disrupted or underexpressed. Cells having MSK2 gene disruptions or underexpressed MSK2 gene sequences may

be used, for example, to screen for agents capable of affecting alternative pathways that compensate for any loss of function attributable to the disruption or underexpression.

In vitro systems may be designed to identify compounds capable of binding the MSK2 gene products. Such compounds may include, but are not limited to, peptides made of D-and/or L-configuration amino acids (in, for example, the form of random peptide libraries; (see e.g., Lam et al., *Nature*, 354:82-4 (1991)), phosphopeptides (in, for example, the form of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang et al., *Cell*, 72:767-78 (1993)), antibodies, and small organic or inorganic molecules. Compounds identified may be useful, for example, in modulating the activity of MSK2 gene proteins, preferably mutant MSK2 gene proteins; elaborating the biological function of the MSK2 gene protein; or screening for compounds that disrupt normal MSK2 gene interactions or themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to the MSK2 gene protein involves preparing a reaction mixture of the MSK2 gene protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the MSK2 gene protein or the test substance onto a solid phase and detecting target protein/test substance complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the MSK2 gene protein may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtitre plates are conveniently utilized. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for MSK2 gene product or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

- 5 Compounds that are shown to bind to a particular MSK2 gene product through one of the methods described above can be further tested for their ability to elicit a biochemical response from the MSK2 gene protein. Agonists, antagonists and/or inhibitors of the expression product can be identified utilizing assays well known in the art.

Antisense, Ribozymes, and Antibodies

- 10 Other agents that may be used as therapeutics include the MSK2 gene, its expression product(s) and functional fragments thereof. Additionally, agents that reduce or inhibit mutant MSK2 gene activity may be used to ameliorate disease symptoms. Such agents include antisense, ribozyme, and triple helix molecules. Techniques for the production and use of such molecules are well known to those of skill in the art.

- 15 Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between the -10 and +10 regions of the MSK2 gene nucleotide sequence of interest, are preferred.

- Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of
20 RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the MSK2 gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see U.S. Patent No. 5,093,246, which is incorporated by reference herein in its entirety. As
25 such within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding MSK2 gene proteins.

- Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribozyme cleavage sites that include the following sequences,
30 GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the MSK2 gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection
35 assays.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

It is possible that the antisense, ribozyme, and/or triple helix molecules described herein may reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by both normal and mutant MSK2 gene alleles. In order to ensure that substantially normal levels of MSK2 gene activity are maintained, nucleic acid molecules that encode and express MSK2 polypeptides exhibiting normal activity may be introduced into cells that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, it may be preferable to coadminister normal MSK2 protein into the cell or tissue in order to maintain the requisite level of cellular or tissue MSK2 gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various well-known modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of

the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Antibodies that are both specific for MSK2 protein, and in particular, the mutant MSK2 protein, and interfere with its activity may be used to inhibit mutant MSK2 gene function. Such
5 antibodies may be generated against the proteins themselves or against peptides corresponding to portions of the proteins using standard techniques known in the art and as also described herein. Such antibodies include but are not limited to polyclonal, monoclonal, Fab fragments, single chain antibodies, chimeric antibodies, antibody mimetics, etc.

In instances where the MSK2 protein is intracellular and whole antibodies are used,
10 internalizing antibodies may be preferred. However, lipofectin liposomes may be used to deliver the antibody or a fragment of the Fab region that binds to the MSK2 gene epitope into cells. Where fragments of the antibody are used, the smallest inhibitory fragment that binds to the target or expanded target protein's binding domain is preferred. For example, peptides having an amino acid sequence corresponding to the domain of the variable region of the antibody that binds to the MSK2
15 protein may be used. Such peptides may be synthesized chemically or produced via recombinant DNA technology using methods well known in the art (*see, e.g.*, Creighton, *Proteins: Structures and Molecular Principles* (1984) W.H. Freeman, New York 1983, *supra*; and Sambrook et al., 1989, *supra*). Alternatively, single chain neutralizing antibodies that bind to intracellular MSK2 gene epitopes may also be administered. Such single chain antibodies may be administered, for example,
20 by expressing nucleotide sequences encoding single-chain antibodies within the target cell population by utilizing, for example, techniques such as those described in Marasco et al., *Proc. Natl. Acad. Sci. USA*, 90:7889-93 (1993).

RNA sequences encoding MSK2 protein may be directly administered to a patient exhibiting disease symptoms, at a concentration sufficient to produce a level of MSK2 protein such that disease
25 symptoms are ameliorated. Patients may be treated by gene replacement therapy. One or more copies of a normal MSK2 gene, or a portion of the gene that directs the production of a normal MSK2 protein with MSK2 gene function, may be inserted into cells using vectors that include, but are not limited to adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes. Additionally, techniques such as those described above
30 may be utilized for the introduction of normal MSK2 gene sequences into human cells.

Cells, preferably autologous cells, containing normal MSK2 gene expressing gene sequences may then be introduced or reintroduced into the patient at positions that allow for the amelioration of disease symptoms.

Pharmaceutical Compositions, Effective Dosages, and Routes of Administration

35 The identified compounds that inhibit target mutant gene expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to treat or ameliorate the disease. A

therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the disease.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, topical, subcutaneous, intraperitoneal, intravenous, intrapleural, intraocular, intraarterial, or rectal administration. It is also contemplated that pharmaceutical compositions may be administered with other products that potentiate the activity of the compound and optionally, may include other therapeutic ingredients.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water

or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives
5 (*e.g.*, methyl or propyl-*p*-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges
10 formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol
15 the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form,
20 *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

25 The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides. Oral ingestion is possibly the easiest method of taking any medication. Such a route of administration, is generally simple and straightforward and is frequently the least inconvenient or unpleasant route of administration from the patient's point of view. However, this involves passing
30 the material through the stomach, which is a hostile environment for many materials, including proteins and other biologically active compositions. As the acidic, hydrolytic and proteolytic environment of the stomach has evolved efficiently to digest proteinaceous materials into amino acids and oligopeptides for subsequent anabolism, it is hardly surprising that very little or any of a wide variety of biologically active proteinaceous material, if simply taken orally, would survive its passage
35 through the stomach to be taken up by the body in the small intestine. The result, is that many

proteinaceous medicaments must be taken in through another method, such as parenterally, often by subcutaneous, intramuscular or intravenous injection.

Pharmaceutical compositions may also include various buffers (*e.g.*, Tris, acetate, phosphate), solubilizers (*e.g.*, Tween, Polysorbate), carriers such as human serum albumin, preservatives (thimerosal, benzyl alcohol) and anti-oxidants such as ascorbic acid in order to stabilize pharmaceutical activity. The stabilizing agent may be a detergent, such as tween-20, tween-80, NP-40 or Triton X-100. EBP may also be incorporated into particulate preparations of polymeric compounds for controlled delivery to a patient over an extended period of time. A more extensive survey of components in pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed., A. R. Gennaro, ed., Mack Publishing, Easton, Pa. (1990).

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Diagnostics

A variety of methods may be employed to diagnose disease conditions associated with the MSK2 gene. Specifically, reagents may be used, for example, for the detection of the presence of MSK2 gene mutations, or the detection of either over- or under- expression of MSK2 gene mRNA.

According to the diagnostic and prognostic method of the present invention, alteration of the wild-type MSK2 gene locus is detected. In addition, the method can be performed by detecting the wild-type MSK2 gene locus and confirming the lack of a predisposition or neoplasia. "Alteration of a wild-type gene" encompasses all forms of mutations including deletions, insertions and point mutations in the coding and noncoding regions. Deletions may be of the entire gene or only a portion of the gene. Point mutations may result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those that occur only in certain tissues, *e.g.*, in tumor tissue, and are not inherited in the germline. Germline mutations can be found in any of a body's tissues and are inherited. If only a single allele is somatically mutated, an early neoplastic state may be indicated. However, if both alleles are mutated, then a late neoplastic state may be indicated. The finding of gene mutations thus provides both diagnostic and prognostic information. a MSK2 gene allele that is not deleted (*e.g.*, that found on the sister chromosome to a chromosome carrying a MSK2 gene

deletion) can be screened for other mutations, such as insertions, small deletions, and point mutations. Mutations found in tumor tissues may be linked to decreased expression of the MSK2 gene product. However, mutations leading to non-functional gene products may also be linked to a cancerous state. Point mutational events may occur in regulatory regions, such as in the promoter of the gene, leading to loss or diminution of expression of the mRNA. Point mutations may also abolish proper RNA processing, leading to loss of expression of the MSK2 gene product, or a decrease in mRNA stability or translation efficiency.

One test available for detecting mutations in a candidate locus is to directly compare genomic target sequences from cancer patients with those from a control population. Alternatively, one could sequence messenger RNA after amplification, *e.g.*, by PCR, thereby eliminating the necessity of determining the exon structure of the candidate gene. Mutations from cancer patients falling outside the coding region of the MSK2 gene can be detected by examining the non-coding regions, such as introns and regulatory sequences near or within the MSK2 gene. An early indication that mutations in noncoding regions are important may come from Northern blot experiments that reveal messenger RNA molecules of abnormal size or abundance in cancer patients as compared to control individuals.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific gene nucleic acid or anti-gene antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings, to diagnose patients exhibiting disease symptoms or at risk for developing disease.

Any cell type or tissue, including brain, cortex, subcortical region, cerebellum, brainstem, olfactory bulb, spinal cord, eye, Harderian gland, heart, lung, liver, pancreas, kidney, spleen, thymus, lymph nodes, bone marrow, skin, gallbladder, urinary bladder, pituitary gland, adrenal gland, salivary gland, skeletal muscle, tongue, stomach, small intestine, large intestine, cecum, testis, epididymis, seminal vesicle, coagulating gland, prostate gland, ovary, uterus and white fat, in which the gene is expressed may be utilized in the diagnostics described below.

DNA or RNA from the cell type or tissue to be analyzed may easily be isolated using procedures that are well known to those in the art. Diagnostic procedures may also be performed *in situ* directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents may be used as probes and/or primers for such *in situ* procedures (*see*, for example, Nuovo, PCR *In Situ* Hybridization: Protocols and Applications, Raven Press, N.Y. (1992)).

Gene nucleotide sequences, either RNA or DNA, may, for example, be used in hybridization or amplification assays of biological samples to detect disease-related gene structures and expression. Such assays may include, but are not limited to, Southern or Northern analyses, restriction fragment length polymorphism assays, single stranded conformational polymorphism analyses, *in situ* hybridization assays, and polymerase chain reaction analyses. Such analyses may reveal both quantitative

aspects of the expression pattern of the gene, and qualitative aspects of the gene expression and/or gene composition. That is, such aspects may include, for example, point mutations, insertions, deletions, chromosomal rearrangements, and/or activation or inactivation of gene expression.

Preferred diagnostic methods for the detection of gene-specific nucleic acid molecules may involve for example, contacting and incubating nucleic acids, derived from the cell type or tissue being analyzed, with one or more labeled nucleic acid reagents under conditions favorable for the specific annealing of these reagents to their complementary sequences within the nucleic acid molecule of interest. Preferably, the lengths of these nucleic acid reagents are at least 9 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:fingerprint molecule hybrid. The presence of nucleic acids from the fingerprint tissue that have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the tissue or cell type of interest may be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtitre plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents are easily removed. Detection of the remaining, annealed, labeled nucleic acid reagents is accomplished using standard techniques well-known to those in the art.

Alternative diagnostic methods for the detection of gene-specific nucleic acid molecules may involve their amplification, *e.g.*, by PCR (the experimental embodiment set forth in Mullis U.S. Patent No. 4,683,202 (1987)), ligase chain reaction (Barany, *Proc. Natl. Acad. Sci. USA*, 88:189-93 (1991)), self sustained sequence replication (Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874-78 (1990)), transcriptional amplification system (Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173-77 (1989)), Q-Beta Replicase (Lizardi *et al.*, *Bio/Technology*, 6:1197 (1988)), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In one embodiment of such a detection scheme, a cDNA molecule is obtained from an RNA molecule of interest (*e.g.*, by reverse transcription of the RNA molecule into cDNA). Cell types or tissues from which such RNA may be isolated include any tissue in which wild-type fingerprint gene is known to be expressed, including, but not limited, to brain, cortex, subcortical region, cerebellum, brainstem, olfactory bulb, spinal cord, eye, Harderian gland, heart, lung, liver, pancreas, kidney, spleen, thymus, lymph nodes, bone marrow, skin, gallbladder, urinary bladder, pituitary gland, adrenal gland, salivary gland, skeletal muscle, tongue, stomach, small intestine, large intestine, cecum, testis, epididymis, seminal vesicle, coagulating gland, prostate gland, ovary, uterus and white fat. A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (*e.g.*, primers) in the reverse transcription and nucleic acid amplification steps of

this method may be chosen from among the gene nucleic acid reagents described herein. The preferred lengths of such nucleic acid reagents are at least 15-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Antibodies directed against wild-type or mutant gene peptides may also be used as disease diagnostics and prognostics. Such diagnostic methods, may be used to detect abnormalities in the level of gene protein expression, or abnormalities in the structure and/or tissue, cellular, or subcellular location of fingerprint gene protein. Structural differences may include, for example, differences in the size, electronegativity, or antigenicity of the mutant fingerprint gene protein relative to the normal fingerprint gene protein.

Protein from the tissue or cell type to be analyzed may easily be detected or isolated using techniques that are well known to those of skill in the art, including but not limited to western blot analysis. For a detailed explanation of methods for carrying out western blot analysis, see Sambrook et al. (1989) *supra*, at Chapter 18. The protein detection and isolation methods employed herein may also be such as those described in Harlow and Lane, for example, (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988)).

Preferred diagnostic methods for the detection of wild-type or mutant gene peptide molecules may involve, for example, immunoassays wherein fingerprint gene peptides are detected by their interaction with an anti-fingerprint gene-specific peptide antibody.

For example, antibodies, or fragments of antibodies useful in the present invention may be used to quantitatively or qualitatively detect the presence of wild-type or mutant gene peptides. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred if the fingerprint gene peptides are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of fingerprint gene peptides. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the fingerprint gene peptides, but also their distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays for wild-type, mutant, or expanded fingerprint gene peptides typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells, or cells that have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying fingerprint gene peptides, and detecting the bound antibody by any of a number of techniques well known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support that is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled gene-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

The terms "solid phase support or carrier" are intended to encompass any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-wild-type or -mutant fingerprint gene peptide antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the gene peptide-specific antibody can be detectably labeled is by linking the same to an enzyme and using it in an enzyme immunoassay (EIA) (Voller, *Ric Clin Lab*, 8:289-98 (1978) ["The Enzyme Linked Immunosorbent Assay (ELISA)", *Diagnostic Horizons* 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, Md.]; Voller et al., *J. Clin. Pathol.*, 31:507-20 (1978); Butler, *Meth. Enzymol.*, 73:482-523 (1981); Maggio (ed.), *Enzyme Immunoassay*, CRC Press, Boca Raton, Fla. (1980); Ishikawa et al., (eds.) *Enzyme Immunoassay*, Igaku-Shoin, Tokyo (1981)). The enzyme that is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes that can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase,

staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect fingerprint gene wild-type, mutant, or expanded peptides through the use of a radioimmunoassay (RIA) (*see, e.g.,* Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Throughout this application, various publications, patents and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents and published

patent specifications referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

The following examples are intended only to illustrate the present invention and should in no way be construed as limiting the subject invention.

5

Examples

Example 1: Generation of Mice Comprising MSK2 Gene Disruptions

To investigate the role of MSK2, disruptions in MSK2 genes were produced by homologous recombination. Specifically, transgenic mice comprising disruptions in MSK2 genes were created. More particularly, as shown in Figure 4, a MSK2-specific targeting construct having the ability to
10 disrupt a MSK2 gene, specifically comprising SEQ ID NO:1, was created using as the targeting arms (homologous sequences) in the construct the oligonucleotide sequences identified herein as SEQ ID NO:3 or SEQ ID NO:4.

The targeting construct was introduced into ES cells derived from the 129/OlaHsd mouse substrain to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females. F2
15 homozygous mutant mice were produced by intercrossing F1 heterozygous males and females.

Example 2: Expression Analysis

RT-PCR Expression. Total RNA was isolated from the organs or tissues from adult C57BL/6 wild-type mice. RNA was DNaseI treated, and reverse transcribed using random primers. The resulting cDNA was checked for the absence of genomic contamination using primers specific to non-
20 transcribed genomic mouse DNA. cDNAs were balanced for concentration using HPRT primers. RNA transcripts were detectable in brain, cortex, subcortical region, cerebellum, brainstem, olfactory bulb, spinal cord, eye, Harderian gland, heart, lung, liver, pancreas, kidney, spleen, thymus, lymph nodes, bone marrow, skin, gallbladder, urinary bladder, pituitary gland, adrenal gland, salivary gland, skeletal muscle, tongue, stomach, small intestine, large intestine, cecum, testis, epididymis, seminal
25 vesicle, coagulating gland, prostate gland, ovary, uterus and white fat.

LacZ Reporter Gene Expression. In general, tissues from 7-12 week old heterozygous mutant mice were analyzed for lacZ expression. Organs from heterozygous mutant mice were frozen, sectioned (10 μ m), stained and analyzed for lacZ expression using X-Gal as a substrate for beta-galactosidase, followed by a Nuclear Fast Red counterstaining.

In addition, for brain, wholemount staining was performed. The dissected brain was cut
30 longitudinally, fixed and stained using X-Gal as the substrate for beta-galactosidase. The reaction was stopped by washing the brain in PBS and then fixed in PBS-buffered formaldehyde.

Wild-type control tissues were also stained for lacZ expression to reveal any background or signals due to endogenous beta-galactosidase activity. The following tissues can show staining in the
35 wild-type control sections and are therefore not suitable for X-gal staining: small and large intestines,

stomach, vas deferens and epididymis. It has been previously reported that these organs contain high levels of endogenous beta-galactosidase activity.

LacZ (beta-galactosidase) expression was detectable in brain, eye, heart, kidney, skin and male reproductive system. LacZ expression was not detected in spinal cord, sciatic nerve, thymus, spleen, lymph nodes, bone marrow, aorta, lung, liver, gallbladder, pancreas, urinary bladder, trachea, larynx, esophagus, thyroid gland, parathyroid gland, pituitary gland, salivary glands, tongue, skeletal muscle and female reproductive system.

Expression:

Brain

10 In wholmount staining faint lacZ expression was detectable in thalamus and hippocampus. On coronal sections faint X-Gal staining was detectable in the pyramidal cell layer of the hippocampus. Thalamus was not present on sections analyzed.

Eyes

Faint lacZ expression was present in the inner nuclear and ganglion cell layer of the retina.

15 Heart

One or two cardiomyocytes in the ventricle of the heart showed lacZ expression.

Kidney

Smooth muscle cells of some blood vessels expressed lacZ faintly.

Skin of the Ear

20 Very faint lacZ expression was detectable in few epithelial cells.

Male Reproductive Systems

Testis

Spermatogenic cells of the seminiferous tubules expressed lacZ weakly. In blood vessels, faint X-Gal staining was detectable.

25 Coagulating Gland

Few epithelial cells of the coagulating gland expressed lacZ at varying levels.

Prostate and Ampullary Gland

Scattered X-Gal staining was detectable in epithelial cells.

Example 3: Physical Examination

30 A complete physical examination was performed on each mouse. Mice were first observed in their home cages for a number of general characteristics including activity level, behavior toward siblings, posture, grooming, breathing pattern and sounds, and movement. General body condition and size were noted as well identifying characteristics including coat color, belly color, and eye color. Following a visual inspection of the mouse in the cage, the mouse was handled for a detailed, 35 stepwise examination. The head was examined first, including eyes, ears, and nose, noting any discharge, malformations, or other abnormalities. Lymph nodes and glands of the head and neck were

palpated. Skin, hair coat, axial and appendicular skeleton, and abdomen were also examined. The limbs and torso were examined visually and palpated for masses, malformations or other abnormalities. The anogenital region was examined for discharges, staining of hair, or other changes. If the mouse defecates during the examination, the feces were assessed for color and consistency.

- 5 Abnormal behavior, movement, or physical changes may indicate abnormalities in general health, growth, metabolism, motor reflexes, sensory systems, or development of the central nervous system.

When compared to age- and gender-matched wild-type control (+/+) mice, one homozygous mutant (-/-) mouse of about 300 days of age exhibited decreased body weight correlated with a thin appearance. The thin appearance and decreased body weight may be due to neoplastic disease.

10 Example 4: Necropsy

Necropsy was performed on mice following deep general anesthesia, cardiac puncture for terminal blood collection, and euthanasia. Body lengths and body weights were recorded for each mouse. The necropsy included detailed examination of the whole mouse, the skinned carcass, skeleton, and all major organ systems. Lesions in organs and tissues were noted during the examination. Designated organs, from which extraneous fat and connective tissue have been removed, were weighed on a balance, and the weights were recorded. Weights were obtained for the following organs: heart, liver, spleen, thymus, kidneys, and testes/epididymides.

- 15 When compared to age- and gender-matched wild-type (+/+) control mice, one homozygous mutant (-/-) mouse of about 300 days of age exhibited a small liver nodule. One homozygous mutant (-/-) mouse of about 49 days of age exhibited decreased body weight, decreased body length, and low organ weights, when compared to wild-type control (+/+) mice (Table 1). One homozygous mutant (-/-) mouse of about 300 days exhibited a decreased body weight, relative to wild-type controls (Table 2).

25

TABLE 1: Necropsy Weights at About 49 Days of Age

Genotype	Gender	Age (days)	Length (cm)	Body Weight (g)	Spleen Weight (g)	Liver Weight (g)	Kidney Weight (g)	Thymus Weight (g)	Heart Weight (g)	Testes + Epididymis Weight (g)
+/+	Male	46	9.5	26.545	0.112	1.211	0.434	0.059	0.196	0.257
+/+	Male	46	9.5	25.853	0.07	1.395	0.434	0.035	0.139	0.225
-/-	Male	47	9.25	25.83	0.096	1.529	0.42	0.071	0.158	0.229
-/-	Male	47	8.5	23.57	0.075	1.316	0.358	0.083	0.136	0.231
-/-	Male	47	9	24.984	0.104	1.665	0.368	0.084	0.171	0.233
-/-	Male	47	6.5	9.602	0.014	0.457	0.173	0.012	0.082	0.129

TABLE 2: Body Weight at About 300 Days of Age

Genotype	Gender	Age (days)	Body Weight (g)
+/+	Female	322	29.44
+/+	Female	322	34.57
-/-	Female	320	22.83

Example 5: Histopathological Analysis

Harvested organs were fixed in about 10% neutral buffered formalin for a minimum of about 48 hours at room temperature. Tissues were trimmed and samples taken to include the major features of each organ. If any abnormalities were noted at necropsy or at the time of tissue trimming, additional sample(s), if necessary, were taken to include the abnormalities so that it is available for microscopic analysis. Tissues were placed together, according to predetermined groupings, in tissue processing cassettes. All bones (and any calcified tissues) were decalcified with a formic acid or EDTA-based solution prior to trimming.

The infiltration of the tissues by paraffin was performed using an automated tissue processor. Steps in the cycle included dehydration through a graded series of ethanols, clearing using xylene or xylene substitute and infiltration with paraffin. Tissues were embedded in paraffin blocks with a standard orientation of specified tissues within each block. Sections were cut from each block at a thickness of about 3-5 μ m and mounted onto glass slides. After drying, the slides were stained with hematoxylin and eosin (H&E) and a glass coverslip was mounted over the sections for examination.

One homozygous mutant (-/-) mouse of about 300 days of age exhibited a carcinoma of undetermined origin. One homozygous mutant (-/-) mouse and one wild-type (+/+) mouse, both about 300 days of age, exhibited carcinoma of the tongue.

Example 6: Behavioral Analysis – Startle Test

The startle test screens for changes in the basic fundamental nervous system or muscle-related functions. The startle reflex is a short-latency response of the skeletal musculature elicited by a sudden auditory stimulus. This includes changes in 1) hearing - auditory processing; 2) sensory and motor processing - related to the auditory circuit and culminating in a motor related output; 3) global sensory changes; and motor abnormalities, including skeletal muscle or motor neuron related changes.

The startle test also screens for higher level cognitive functions. The startle reflex can be modulated by negative affective states like fear or stress. The cognitive changes include: 1) sensorimotor processing such as sensorimotor gating changes related to schizophrenia; 2) attention disorders; 3) anxiety disorders; and 4) thought disturbance disorders.

The mice were tested in a San Diego Instruments SR-LAB sound response chamber. Each mouse was exposed to 9 stimulus types that were repeated in pseudo-random order ten times during

the course of the entire 25-minute test. The stimulus types in decibels were: p80, p90, p100, p110, p120, pp80, p120, pp90, p120, pp100, and p120; where p=40 msec pulse, pp=20 msec prepulse. The length of time between a prepulse and a pulse was 100 msec (onset to onset). The mean Vmax of the ten repetitions for each trial type was computed for each mouse.

- 5 When compared to age- and gender-matched wild-type control mice, homozygous mutant mice exhibited significantly increased startle responses to a sonic pulse of about 100dB (Table 3).

TABLE 3: Startle Test

Genotype	Gender	Age (days)	Average Highest voltage P100
+/+	Male	77	60.9
+/+	Male	73	20.8
+/+	Male	73	36.6
+/+	Male	76	75.1
+/+	Male	80	96.7
+/+	Male	75	70.8
-/-	Male	81	165.4
-/-	Male	77	93.2
-/-	Male	77	160.1
-/-	Male	73	74.2
-/-	Male	73	161.6
-/-	Male	78	126
-/-	Male	80	120
-/-	Male	75	78.6

Example 7: Aging Metrics

- 10 Body weights and body lengths were measured for mice at about 49, 90, 180 and 300 days of age.

When compared to age- and gender-matched wild-type control mice homozygous mice exhibited a decreased body weight and body weight to body length ratio at about 49, about 90 and about 180 days of age (Table 4).

TABLE 4: Aging Measurements

Genotype	Gender	Age (days)	Mouse ID	Body Weight (g)	Body Length (g)	Body Weight/ Body Length Ratio
+/+	Male	50	149842	26.18	9.50	2.76
+/+	Male	89	149842	33.51	10.00	3.35
+/+	Male	186	149842	42.53	10.50	4.05
+/+	Male	302	149842	55.94	11.25	4.97
+/+	Male	50	149845	26.76	9.75	2.74
+/+	Male	89	149845	34.15	10.00	3.42
+/+	Male	186	149845	39.58	10.50	3.77
+/+	Male	302	149845	47.36	11.00	4.31
+/+	Male	48	188894	27.40	10.00	2.74
+/+	Male	91	188894	34.55	10.50	3.29
+/+	Male	182	188894	42.50	10.75	3.95
+/+	Male	50	198327	27.99	10.00	2.80
+/+	Male	92	202479	40.81	10.50	3.89
+/+	Male	179	202479	45.78	10.00	4.58
-/-	Male	50	236785	24.48	10.00	2.45
-/-	Male	89	236785	26.96	10.50	2.57
-/-	Male	50	149834	22.36	9.50	2.35
-/-	Male	92	149834	26.76	10.00	2.68
-/-	Male	184	149834	35.56	10.75	3.31
-/-	Male	50	149841	27.51	10.00	2.75
-/-	Male	89	149841	32.58	10.25	3.18
-/-	Male	186	149841	37.33	11.00	3.39
-/-	Male	302	149841	47.86	11.50	4.16

As is apparent to one of skill in the art, various modifications of the above embodiments can be made without departing from the spirit and scope of this invention. These modifications and variations are within the scope of this invention.

CLAIMS

We claim:

1. A transgenic mouse comprising a disruption in a MSK2 gene.
2. A transgenic mouse comprising a disruption in a MSK2 gene, wherein there is no native expression of endogenous MSK2 gene.
3. The transgenic mouse of claim 2, wherein the disruption is heterozygous.
4. The transgenic mouse of claim 2, wherein the disruption is homozygous.
5. The transgenic mouse of claim 4, wherein the transgenic mouse exhibits a growth abnormality.
6. The transgenic mouse of claim 5, wherein the growth abnormality is selected from the group consisting of decreased body weight, decreased body length, decreased body weight to body weight ratio, thin appearance and decreased organ weight.
7. The transgenic mouse of claim 6, wherein the decreased organ weight is selected from the group consisting of decreased spleen weight, decreased liver weight, decreased thymus weight, decreased kidney weight, decreased heart weight and decreased testes weight.
8. The transgenic mouse of claim 4, wherein the transgenic mouse exhibits a histopathological abnormality.
9. The transgenic mouse of claim 8, wherein the histopathological abnormality is selected from the group consisting of liver nodule and carcinoma.
10. The transgenic mouse of claim 9, wherein the carcinoma is a carcinoma of the tongue.
11. The transgenic mouse of claim 4, wherein the transgenic mouse exhibits an increased startle response in a startle test.
12. The transgenic mouse of claim 11, wherein the increased startle response is consistent with a symptom associated with human anxiety.
13. A method of producing a transgenic mouse comprising a disruption in a MSK2 gene, the method comprising:
 - (a) providing a murine stem cell comprising a disruption in a MSK2 gene; and
 - (b) introducing the murine stem cell into a pseudopregnant mouse, wherein the pseudopregnant mouse gives birth to a transgenic mouse.
14. The transgenic mouse produced by the method of claim 13.
15. A targeting construct comprising:
 - (a) a first polynucleotide sequence homologous to at least a first portion of a MSK2 gene;
 - (b) a second polynucleotide sequence homologous to at least a second portion of a MSK2 gene; and
 - (c) a selectable marker.

16. A cell comprising a disruption in a MSK2 gene, the disruption produced using the targeting construct of claim 15.
17. A cell derived from the transgenic mouse of claim 2.
18. A cell comprising a disruption in a MSK2 gene.
19. The cell of claim 18, wherein the cell is a stem cell.
20. The cell of claim 19, wherein the stem cell is an embryonic stem cell.
21. The cell of claim 20, wherein the embryonic stem cell is a murine cell.
22. A method of identifying an agent that modulates a phenotype selected from the group consisting of decreased body weight, decreased body length, decreased body weight to body weight ratio, thin appearance and decreased organ weight, the method comprising:
 - (a) contacting a test agent with a MSK2 serine/threonine kinase; and
 - (b) determining whether the agent modulates the MSK2 serine/threonine kinase.
23. A method of identifying an agent that modulates a phenotype selected from the group consisting of liver nodule and carcinoma, the method comprising:
 - (a) contacting a test agent with a MSK2 serine/threonine kinase; and
 - (b) determining whether the agent modulates the MSK2 serine/threonine kinase.
24. A method of identifying an agent that modulates an increased startle response in a startle test, the method comprising:
 - (a) contacting a test agent with a MSK2 serine/threonine kinase; and
 - (b) determining whether the agent modulates the MSK2 serine/threonine kinase.
25. A method of identifying an agent that modulates a phenotype selected from the group consisting of decreased body weight, decreased body length, decreased body weight to body weight ratio, thin appearance and decreased organ weight, the method comprising:
 - (a) administering a test agent to an animal exhibiting a phenotype selected from the group consisting of decreased body weight, decreased body length, decreased body weight to body weight ratio, thin appearance and decreased organ weight; and
 - (b) determining whether the agent modulates the phenotype.
26. A method of identifying an agent that modulates a phenotype selected from the group consisting of liver nodule and carcinoma, the method comprising:
 - (a) administering a test agent to an animal exhibiting a phenotype selected from the group consisting of liver nodule and carcinoma; and
 - (b) determining whether the agent modulates the phenotype.
27. A method of identifying an agent that modulates an increased startle response in a startle test, the method comprising:

- (a) administering a test agent to an animal exhibiting an increased startle response in a startle test; and
 - (b) determining whether the agent modulates the phenotype.
28. A method of identifying a potential therapeutic agent for the treatment of anxiety, the method comprising:
- (a) administering the potential therapeutic agent to a transgenic mouse comprising a disruption in a MSK2 gene; and
 - (b) determining whether the potential therapeutic agent modulates a symptom consistent with anxiety, wherein modulation of the symptom identifies a potential therapeutic agent for the treatment of anxiety.
29. A method of identifying a potential therapeutic agent for the treatment of anxiety, the method comprising:
- (a) contacting the potential therapeutic agent with a MSK2 serine/threonine kinase;
 - (b) determining whether the agent modulates the MSK2 serine/threonine kinase, wherein modulation of the MSK2 serine/threonine kinase identifies a potential therapeutic agent for the treatment of anxiety.
30. A method of evaluating a potential therapeutic agent capable of affecting a condition associated with a mutation in a MSK2 gene, the method comprising:
- (a) administering the potential therapeutic agent to a transgenic mouse comprising a disruption in a MSK2 gene; and
 - (b) evaluating the effects of the agent on the transgenic mouse.
31. A method of evaluating a potential therapeutic agent capable of affecting a condition associated with a mutation in a MSK2 gene, the method comprising:
- (a) contacting the potential therapeutic agent with a MSK2 serine/threonine kinase;
 - (b) evaluating the effects of the agent on the MSK2 serine/threonine kinase.
32. A method of determining whether an agent modulates a MSK2 serine/threonine kinase, the method comprising:
- (a) providing a first preparation derived from the mouse of claim 2;
 - (b) providing a second preparation derived from a wild-type mouse;
 - (c) contacting a test agent with the first and second preparations; and
 - (d) determining whether the agent modulates the first and second preparations, wherein modulation of the second preparation but not the first preparation indicates that the agent modulates the MSK2 serine/threonine kinase.
33. A therapeutic agent for treating anxiety, wherein the agent modulates a MSK2 serine/threonine kinase.

34. A therapeutic agent for treating anxiety, wherein the agent is an agonist of a MSK2 serine/threonine kinase.
35. A pharmaceutical composition comprising a MSK2 gene or a MSK2 serine/threonine kinase.
36. A method of preparing a pharmaceutical composition for a condition associated with a function of a MSK2 serine/threonine kinase, the method comprising:
 - (a) identifying a compound that modulates the MSK2 serine/threonine kinase;
 - (b) synthesizing the identified compound; and
 - (c) incorporating the compound into a pharmaceutical carrier.
37. Phenotypic data associated with a transgenic mouse comprising a disruption in a MSK2 gene, wherein the phenotypic data is in an electronic database.

1/4

GTAACCGGCGCCAGCCGTAGCCCCAGCCGCGCGGCCCCAGCGACCCGCGCCCATGGGAGACGAGGATG
AGGACGAGGGCTGCGCCGTGGAGCTGCAGATCACCGAAGCCAACCTCACCGGGCATGAGGAGAAGGTG
AGCGTGGAGAACTTCGCGCTGCTCAAGGTGCTGGGCACGGGAGCCTATGGGAAGGTGTTCTGGTGCG
GAAGACGGGTGGGCACGACGCGGGCAAGCTCTATGCCATGAAGGTGCTACGCAAGGCGGCGTTGGTGCG
AGCGCGCAAGACACAGGAGCATACCCGACCGAAGCTCGGTGCTGGAGCTGGTTCCGCAAGCACCCC
TTCTGGTCACTGCACTACGCTTCCAGACGGATGCCAAGCTGCACCTCATCTGGACTACGTGAG
CGGTGGTGAGATGTTCACTCACTCTACCAGCGCCAGTACTTCAAGGAGGCTGAGGTTTCGAGTGTATG
GGGCGGAGATTGTGCTGGCCCTGGAACACCTGCACAAGCTGGGTATCATCTACCGGGACCTGAAGCTG
GAGAACGTCTTACTTGACTCAGAAGGTCACATCGTCTTACAGACTTTGGGCTGAGCAAGGAGTTCTT
GACGGAGGAGAAAGAGCGGACCTTCTCCTTCTGTGGCACAATCGAGTACATGGCTCCCGAAATCATCC
GAAGCAAGGCTGGACATGGCAAGGCTGTGGACTGGTGGAGCCTGGGTATCCTGCTCTTCGAGCTGCTG
ACAGGGGCTCACCTTCACTTGAGGGGAGAGGAACACTCAGGCTGAGGTGTCCCGACGGATCTT
GAAGTGCTCCCCCTCCCTTCCCTCTCCGGATTGGGCTGTGGCACAGGACCTGCTACAGCGGCTGCTGT
GCAAGGACCTTAAGAAGAGGTTGGGCGCAGGTCCCCAGGGTGCGCAGGAAGTCAAGAGTCAACCCCTTC
TTCCAGGGTCTGGACTGGTGGGCTCTGGCTGCCAGAAAGATCCAGCCCCATTCCGGCCCCAGATCCG
CTCAGAGCTGGATGTGGGAATTTTGGCGAGGAATTCACCCGGCTGGAGCCCGTCTACTCCCTGCGAG
GCAGCCCTCCACCTGGGGACCTTCGGATCTTTCAGGGATACTCCTTCGTGGCTCCGTCCATCCTCTTT
GACCACAACAATGCAGTGATGGCTGATGTACTGCAGGCACCGGGTGCCGGATACAGGCCCGGCAGGGC
AGCAGTTGCCAGGAGTGCCATGATGCAGGACTCGCCTTTCTTCCAGCAGTACGAACTGGACCTTCGGG
AGCCAGCGCTGGGGCAGGGCAGCTTCTCTGTGTGTCTGGAGATGTAGGCAGCGCCAGAGCGGCCAGGAG
TTTGCTGTCAAGATCCTCAGCCGCAGGCTGGAGGAGAACACTCAGCGAGAGGTGGCTGCTCTTCGCTT
GTGCCAGTCACACCCCAACGTGGTGAATCTGCATGAGGTGCTTCATGACCAGCTACACACTTACCTGG
TCCTGGAGTTGCTGCGAGGCGGAGAGCTATTGGAACACATCCGCAAGAAGCGGCTCTTCAGCGAGTCG
GAGGCCAGCCAGATCCTTCGGAGCCTGGTTTCGGCCGTGAGCTTCATGCACGAGGAGGCAGGCGTGGT
GCACCGCGACCTGAAACCCGAGAACATCTTGTACGCGGACGACACTCCCGGGGCCCCGGTGAAGATCA
TCGACTTCGGGTTCCGCGGACTGCGGCCCCAGAGCCCGGCAGAGCCCATGCAGACTCCTTGCTTCACA
CTGCAGTACGCTGCACCCGAGCTGCTGGCACAGCAGGGCTACGATGAGTCTGCGATCTATGGAGCCT
GGGTGTCAATCTGTACATGATGCTGTCTGGCCAGGTTCCCTTCCAAGGGGCTCCGGCCAGGGTGGAC
AGAGTCAGGCAGCTGAGATCATGTGCAAGATCCGTGAAGGGCGCTTCTCCCTGGACGGGGAAGCCTGG
CAAGGTGTGTGCGAGGAAGCCAAGGAGCTGGTCCGAGGGCTACTGACAGTCGACCCCGCCAAGCGGCT
GAAGCTGGAGGGGCTGCGTAGCAGCTCGTGGCTTCAGGACGGCAGCGCGCGCTCCTCGCCCCGCTCC
GCACGCCGGATGTGCTGGAGTCTCTGGGCCAGCTGTGCGTTCCGGGCTCAATGCCACTTTCATGGCG
TTCAACCGAGGCAAGCGCGAGGGCTTCTTTCTCAAGAGTGTAGAGAATGCGCCTCTGGCCAAGAGGCG
CAAGCAGAAGCTCCGGAGCGCGGCGCCTCACGCAGAGGCTCCCCAGTGCCTGCCTCCTCCGGTCCG
TACCAGCCTCTGCCGCTAAGGGGACAACTCGCCGAGCCAACGGCCCCCTTGTCCCCCTTCTAGCTCCCC
CCACTGTGACCCCTTACCTTTAGGAGCTCCGATCCGGGAGCCTGGTGTGCCACCAGACTTTTGCCA
GCGGCTCCCACTCTGATCCCTAGGAATTGCCCTCCCCCTCCACACCCACTTCCAGACAGAGCAGAAGTA
TTTTTATAAGCAGAGATTTTAAAGTGTCTTACTAGATAAGAGTTGGGGACACAGAGAAGCAACTG
TTGTGAGCAAAGTGGAGCTTGGGGCTCCCTCGCCCGATCCCAATGCTGCCCCATCAGGAAGAGAGAAG
GTCCTTTCCAGGAGCAACTCTGCTAAGAAAGGGCATCCTATCCTGTTCTAAGTACCCAGTTGCCATG
GAGAAGAAATCAGGAGCCCGTATACTGTGCTTCCCTGGGACTTTGCCCCCTAGGAGGGGACACCCATCA
ATGTCACTTTATGGACCGTGTGCAATGATGTCCACCAAGACCTGTGTTGGGAATGAGAGAGAGAGAG
ACCTTGAGGGACCTCTAGAGCATTCTGAGATACATCACTTTTGTCAACGGCTCCCTTAGTGGGGTTA
AGGAAGAAGGCAGGTGCTCCCCAGGGGGAGCCCCCTTCTCACTCACCCATTCTCATGGGCACAGCTG
CTTCTGGCTGCAGGTGGAGCCTTAGGGTCTGGGCTAGGCATCCACAGTCACTGCCTCAGCCCCCTCTA
GGCTGTGCCTTTGACTTTAAATAAACTCCACCCAGTGCTGCAAAAAAAAAAAAAAAAAA (SEQ ID
NO:1)

FIGURE 1

2/4

MGDEDEDEGCVELQITEANLTGHEEKVSVENFALLKVLGTGAYGKVFLVRKTGGHDAGKLYAMKVL
KAALVQRAKTQEHTRTERSVLELVRQAPFLVTLHYAFQTDAKLHLILDYVSGGEMFTHLYQRQYFKEA
EVRVYGGGEIVLALHLHLKLGIIYRDLKLENVLLDSEGHIVLTDGLSKEFLTEEKERTFSFCGTIEYM
APEIIRSKAGHGKAVDWWSLGILLFELLTGASPFTEGERNTQAEVSRILKCSPPFPLRIGPVAQDL
LQRLLCCKDPKKRLGAGPQGAQEVKSHPFQGLDWWALAARKIPAPFRPQIRSELDVGNFAEEFTRLEP
VYSPAGSPPPGDPRIFQGYSFVAPSILFDHNNVMAVLDLQAPGAGYRPGRAAVARSAMMDSPPFQOY
ELDLREPALGQGSFSVCRRRCRQRQSGQEFVAVKILSRRLLEENTQREVAALRLCQSHPNVNLHEVLHDQ
LHTYLVLLELLRGGELLEHIRKKRLFSESEASQILRSLVSAVSFMHEEAGVVHRDLKPNILYADDTPG
APVKIIDFGFARLRPQSPAEPMTPCFTLQYAAPELLAQQGYDESCDLWSLGVILYMLLSGQVPFQGA
SGQGGQSQAAEIMCKIREGRFSLDGEAWQGVSEEAKELVRGLLTVDPAKRLKLEGLRSSSWLQDGSAR
SSPPLRTPDVLESSGPAVRSGLNATFMAFNRGKREGFFLKSVENAPLAKRRKQKLRSAAASRRGSPVP
ASSGRLPASAAGTTRRANGPLSPS (SEQ ID NO:2)

FIGURE 2

3/4

underlined = deleted in targeting construct**BOLD** = sequence flanking Neo insert in targeting construct

GTAACCGGCGCCAGCCGTAGCCCCAGCCGCGCGCCCCAGCGACCCGCGCCATGGGAGACGAGGATGAGGACG
 AGGGCTGCGCCGTGGAGCTGCAGATCACCAGCAAGCCAACCTCACCGGGCATGAGGAGAAGGTGAGCGTGGAGAAC
 TTCGCGCTGCTCAAGGTGCTGGGCACGGGAGCCTATGGGAAGGTGTTCTTGGTGCAGCGCGCAAGACACAGGAGCATA
CGCGGGCAAGCTCTATGCCATGAAGGTGCTACGCAAGCGCGCGTTGGTGCAGCGCGCAAGACACAGGAGCATA
CCCGCACCGAAGCTCGGTGCTGGAGCTGGTTCGCCAAGCACCCCTTCTGGT**CACACTGC**ACTACGCCTTCCAG
ACGGATGCCAAGCTGCACCTCATCTGGACTACGTGAGCGGTGGTGAGATGTTCACTCACCTCTACCAGCGCCA
 GTACTTCAAGGAGGCTGAGGTTGAGTGTATGGGGCGGAGATTGTGCTGGCCCTGGAACACCTGCACAAGCTGG
 GTATCATCTACCGGGACCTGAAGCTGGAGAACGTCCTTACTTGACTCAGAAGGTACATCGTCCTTACAGACTTT
 GGGCTGAGCAAGGAGTTCCTGACGGAGGAGAAAGAGCGGACCTTCTCCTTCTGTGGCACAATCGAGTACATGGC
 TCCCGAAATCATCCGAAGCAAGGCTGGACATGGCAAGGCTGTGGACTGGTGGAGCCTGGGTATCCTGCTCTTCG
 AGCTGCTGACAGGGGCCCTCACCTTTCACACTGGAGGGAGAGAGGAACACTCAGGCTGAGGTGTCCCGACGGATC
 TTGAAGTGCTCCCTCCCTTCCCTCTCCCGATTGGGCCTGTGGCACAGGACCTGTACAGCGGCTGTGTGCAA
 GGACCCTAAGAAGAGGTTGGGCGCAGGTCCCAGGGTGCGCAGGAAGTCAAGAGTCACCCCTTCTTCCAGGGTCT
 TGGACTGGTGGGCTCTGGCTGCCAGAAAGATCCCAGCCCCATTCCGGCCCCAGATCCGCTCAGAGCTGGATGTG
 GGGAAATTTGCGGAGGAATTCACCCGGCTGGAGCCGCTCTACTCCCTGCAGGCAGCCCTCCACCTGGGGACCC
 TCGGATCTTTTACGGGATCTCCTTCTGTGGCTCCGTCCATCTCTTTGACCACAACAATGCAGTATGGCTGATG
 TACTGCAGGCACCGGGTGCCGGATACAGGCCCGGCAGGGCAGCAGTTGCCAGGAGTGCCATGATGCAGGACTCG
 CCTTCTTCCAGCAGTACGAACCTGGACCTTCGGGAGCCAGCGCTGGGGCAGGGCAGCTTCTCTGTGTGTCGGAG
 ATGTAGGCAGCGCCAGAGCGGCCAGGAGTTTGTGTCAAGATCCTCAGCCGCAGGCTGGAGGAGAACACTCAGC
 GAGAGGTGGCTGCTCTTCGCCTGTGCCAGTCAACCCCCAACGTGGTGAATCTGCATGAGGTGCTTCATGACCAG
 CTACACACTTACCTGGTCTTGGAGTTGCTGCGAGGCGGAGAGCTATTGGAACACATCCGCAAGAAGCGGCTCTT
 CAGCGAGTCGGAGGCCAGCCAGATCCTTCGGAGCCTGGTTTCGGCCGTGAGCTTCATGCACGAGGAGCAGGCG
 TGGTGACCCGACCTGAAACCCGAGAATCTTTGTACGGGACGACACTCCCGGGGCCCCGGTGAAGATCATC
 GACTTCGGGTTCGCGCGACTGCGGCCCCAGAGCCCCGAGAGCCCATGCAGACTCCTTGCTTCACACTGCAGTA
 CGCTGCACCCGAGCTGCTGGCACAGCAGGGCTACGATGAGTCTTGCAGTCTATGGAGCCTGGGTGTCTATTCTGT
 ACATGATGCTGTCTGGCCAGGTTCCCTTCCAAGGGGCTCCGGCCAGGGTGGACAGAGTCAGGCAGCTGAGATC
 ATGTGCAAGATCCGTGAAGGGCGCTTCTCCCTGGACGGGGAAGCCTGGCAAGGTGTGTGCGGAGGAAGCCAAGGA
 GCTGGTCCGAGGGCTACTGACAGTCGACCCCGCAAGCGGCTGAAGCTGGAGGGGCTGCGTAGCAGCTCGTGGC
 TTCAGGACGGCAGCGCGCGCTCTCGCCCCGCTCCGCACGCCGATGTGCTGGAGTCTCTGGGCCAGCTGTG
 CGTTCCGGGCTCAATGCCACTTTTCATGGCGTTCAACCGAGGCAAGCGCGAGGGCTTCTTCTCAAGAGTGTAGA
 GAATGCGCCTCTGGCCAAGAGGCGCAAGCAGAAGCTCCGGAGCGCGGCCCTCACGCAGAGGCTCCCCAGTGC
 CTGCCCTCTCCGGTCGCCCTACCAGCCTCTGCCGCTAAGGGGACAATCGCCGAGCCAACGGCCCCCTTGTCCCCCT
 TCTAGCTCCCGCCACTGTGACCCCCCTTACCTTTAGGAGCTCCGATCCGGGAGCCTGGTGTGCCACCAGACTT
 TTGCCAGCGGCTCCCACTCTGATCCCTAGGAATTGCCCTCCCCCTCCACACCCACTTCCAGACAGAGCAGAAGTA
 TTTTATAAGCAGAGATTTTAAAGTGTCTTACTAGATAAGAGTTGGGGACACAGAGAAGCAACTGTTGTGA
 GCAAAGTGGAGCTTGGGGCTCCCTCGCCCCGATCCCAATGCTGCCCATCAGGAAGAGAGAAGGTCCCTTCCAGG
 AGCAACTCTGCTAAGAAAGGGCATCCTATCTGTTCCTAAGTACCCAGTTGCCATGGAGAAGAAATCAGGAGCC
 CGTATACTGTCTTCCCTGGGACTTTGCCCTTAGGAGGGGACACCCATCAATGTCACTTTATGGACCGTGTGCA
 ATGATGTCCACAAAGACCTGTGTTGGGAATGAGAGAGAGAGACCCCTGAGGGACCTTAGAGCATTTCTGAG
 ATACATCACTTTTGTCAACGGCTCCCTTAGTGGGTTAAGGAAGAAGGCAGGTGCTCCCCAGGGGGAGCCCCCTT
 TCTCACTACCCATTCTCATGGGCACAGTGTCTTGTGGCTGCAGGTGGAGCCTTAGGGTTCTGGGCTAGGCAT
 CCACAGTCACTGCCTCAGCCCCCTTAGGCTGTGCCTTTGACTTTAAATAAAACTCCACCCAGTGCTGCAAAAA
 AAAAAAAAAA (SEQ ID NO:1)

FIGURE 3

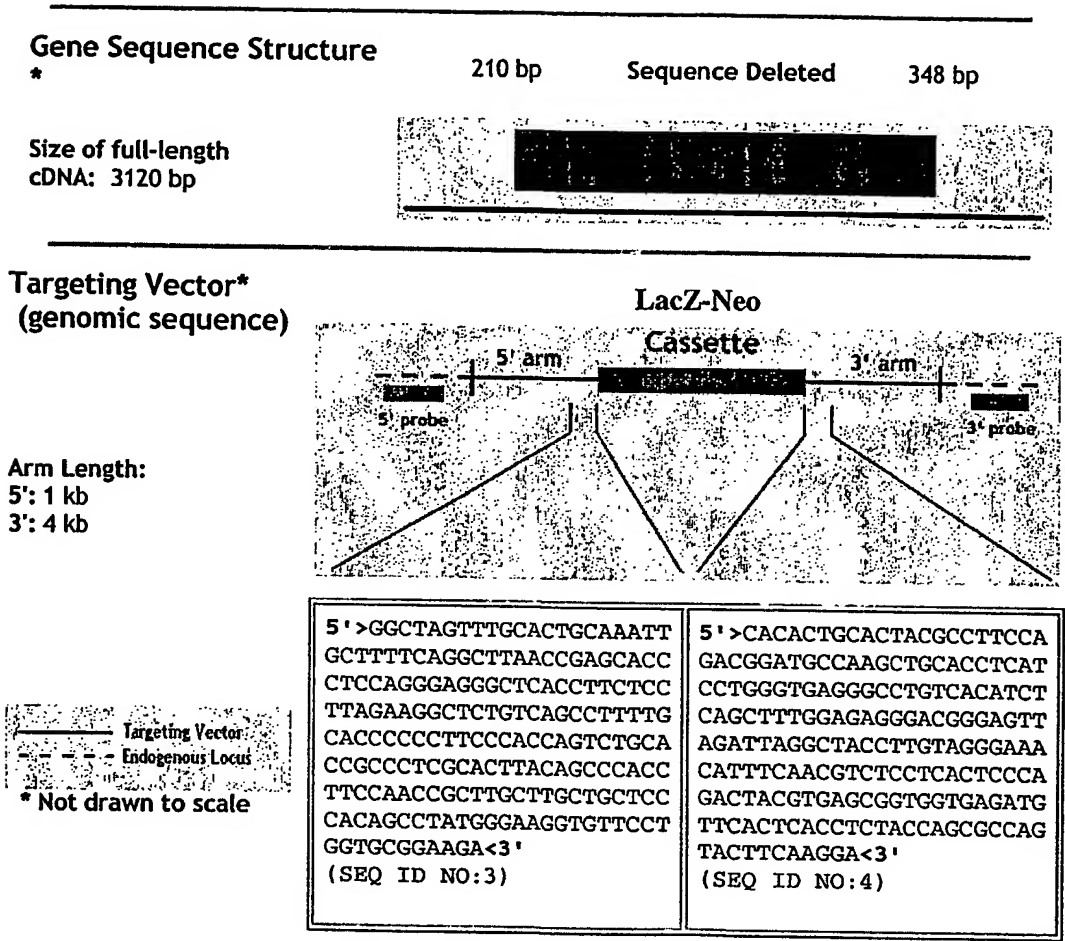


FIGURE 4

SEQUENCE LISTING

<110> Deltagen, Inc.

<120> TRANSGENIC MICE CONTAINING MSK2
SERINE/THREONINE KINASE GENE DISRUPTIONS

<130> 523 PCT

<150> US 60/280,370

<151> 2001-03-29

<160> 4

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 3120

<212> DNA

<213> Mus musculus

<400> 1

```

gtaaccggcg ccagccgtag cccagccgc gcgccccag cgaccgcgc ccatgggaga 60
cgaggatgag gacgagggtc gcgccgtgga gctgcagatc accgaagcca acctcaccgg 120
gcatgaggag aaggtgagcg tggagaactt cgcgctgctc aaggtgctgg gcacgggagc 180
ctatgggaag gtgttcctgg tgcggaagac ggggtggcac gacgcgggca agctctatgc 240
catgaagggt ctacgcaagg cggcggttgg gcagcgcgcg aagacacagg agcatacccg 300
caccgaacgc tcggtgctgg agctgggttcg ccaagcacc ttcctggtca cactgcaacta 360
cgccctccag acggatgcc aagctgcacct catcctggac tacgtgagcg gtggtgagat 420
gttcactcac ctctaccagc gccagttact caaggaggct gaggttcgag tgtatggggg 480
cgagattgtg ctggccctgg aacacctgca caagctgggt atcatctacc gggacctgaa 540
gctggagaac gtcttacttg actcagaagg tcacatcgct cttacagact ttgggctgag 600
caaggagtct ctgacggagg agaaagagcg gaccttctcc ttctgtggca caatcgagta 660
catggctccc gaaatcatcc gaagcaaggc tggacatggc aaggctgtgg actggtggag 720
cctgggtatc ctgctcttcg agctgctgac aggggcctca cccttcacac tggagggaga 780
gaggaacact caggctgagg tgtcccgacg gatcttgaag tgctccctc ccttccctct 840
ccggattggg cctgtggcac aggacctgct acagcggctg ctgtgcaagg accctaagaa 900
gaggttgggc gcaggctccc aggggtgcga ggaagtcaag agtcacccct tcttccaggg 960
tctggactgg tgggctctgg ctgccagaaa gatccagcc ccattccggc cccagatccg 1020
ctcagagctg gatgtgggga attttgcgga ggaattcacc cggctggagc ccgtctactc 1080
ccctgcaggc agccctccac ctggggaccc tcggatcttt cagggatact ccttcgtggc 1140
tccgtccatc ctctttgacc acaacaatgc agtgatggct gatgtactgc aggcaccggg 1200
tgccggatac aggcceggca gggcagcagt tgccaggagt gccatgatgc aggactcgcc 1260
tttcttccag cagtacgaac tggaccttcg ggagccagcg ctggggcagg gcagcttctc 1320
tgtgtgtcgg agatgtaggc agcgccagag cggccaggag ttgctgttca agatcctcag 1380
ccgaggctg gaggaagaaca ctcagcgaga ggtggctgct cttcgctgt gccagtcaca 1440
cccacacgtg gtgaatctgc atgaggtgct tcatgaccag ctacacactt acctggctct 1500
ggagtgtcgt cgaggcggag agctattgga acacatccgc aagaagcggc tcttcagcga 1560
gtcggaggcc agccagatcc ttcggagcct ggtttcggcc gtgagcttca tgcacgagga 1620
ggcaggcgtg gtgcaccgcy acctgaaacc cgagaacatc ttgtacgcgg acgacactcc 1680
cggggccccc gtgaagatca tcgacttcgg gttcgcgcga ctgcggcccc agagcccgcc 1740
agagcccatg cagactcctt gcttcacact gcagtagcgt gcacccgagc tgctggcaca 1800
gcagggtac gatgagtcct gcgactatg ggcctgggt gtcattctgt acatgatgct 1860
gtctggccag gttcccttcc aaggggcctc cggccagggt ggacagagtc aggcagctga 1920
gatcatgtgc aagatccgtg aagggcgctt ctccctggac ggggaagcct ggcaagggtg 1980
gtcggaggaa gccaaaggag tgggtccgag gctactgaca gtcgaccccc ccaagcggct 2040
gaagctggag gggctgcgta gcagctcggt gcttcaggac ggcagcgcg gctcctcgcc 2100
cccgtccgc acgcccgatg tgctggagtc ctctgggcca gctgtgcgtt ccggggtcaa 2160
tgccactttc atggcggttca accgaggcaa gcgcgagggc ttctttctca agagtgtaga 2220

```



```

gaatgcgcct ctggccaaga ggcgcaagca gaagctccgg agcgcggccg cctcacgcag 2280
aggctcccca gtgcctgcct cctccggtcg cctaccagcc tctgccgcta aggggacaac 2340
tcgccgagcc aacggcccct tgtccccttc ctactgtgac ccccttacct 2400
ttaggagctc cgatccggga gcctgggtgt gccaccagac ttttgccagc ggctcccact 2460
ctgatcccta ggaattgccc tcccctccac acccacttcc agacagagca gaagtatttt 2520
tataagcaga gatTTTTTaa aagtgtctta ctagataaga gttggggaca cagagaagca 2580
actgttgtga gcaaagtggg gcttggggct ccctcgcccg atcccaatgc tgcccatca 2640
ggaagagaga aggtcctttc caggagcaac tctgctaaga aagggcatcc tatcctgttc 2700
ctaagtaccc agttgccatg gagaagaaat caggagcccg tatactgtgc ttccctggga 2760
ctttgcccct aggaggggac acccatcaat gtcactttat ggaccgtgtg caatgatgtc 2820
caccaaagac ctgtgttggg aatgagagag agagagaccc tgagggacct ctagagcatt 2880
tctgagatac atcacttttg tcaacggctc ccctagtggg gttaaggaag aaggcagggtg 2940
ctcccagggg ggagcccctt tctcactcac ccattcctca tgggcacagc tgcttctggc 3000
tgcagggtga gccttagggg tctgggctag gcacccacag tcactgcctc agcccctcta 3060
ggctgtgcct ttgactttaa aataaaaact caccagtgcc tgcaaaaaaa aaaaaaaaaa 3120

```

<210> 2

<211> 773

<212> PRT

<213> Mus musculus

<400> 2

```

Met Gly Asp Glu Asp Glu Asp Glu Gly Cys Ala Val Glu Leu Gln Ile
  1              5              10              15
Thr Glu Ala Asn Leu Thr Gly His Glu Glu Lys Val Ser Val Glu Asn
              20              25              30
Phe Ala Leu Leu Lys Val Leu Gly Thr Gly Ala Tyr Gly Lys Val Phe
              35              40              45
Leu Val Arg Lys Thr Gly Gly His Asp Ala Gly Lys Leu Tyr Ala Met
              50              55              60
Lys Val Leu Arg Lys Ala Ala Leu Val Gln Arg Ala Lys Thr Gln Glu
              65              70              75              80
His Thr Arg Thr Glu Arg Ser Val Leu Glu Leu Val Arg Gln Ala Pro
              85              90              95
Phe Leu Val Thr Leu His Tyr Ala Phe Gln Thr Asp Ala Lys Leu His
              100             105             110
Leu Ile Leu Asp Tyr Val Ser Gly Gly Glu Met Phe Thr His Leu Tyr
              115             120             125
Gln Arg Gln Tyr Phe Lys Glu Ala Glu Val Arg Val Tyr Gly Gly Glu
              130             135             140
Ile Val Leu Ala Leu Glu His Leu His Lys Leu Gly Ile Ile Tyr Arg
              145             150             155             160
Asp Leu Lys Leu Glu Asn Val Leu Leu Asp Ser Glu Gly His Ile Val
              165             170             175
Leu Thr Asp Phe Gly Leu Ser Lys Glu Phe Leu Thr Glu Glu Lys Glu
              180             185             190
Arg Thr Phe Ser Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu Ile
              195             200             205
Ile Arg Ser Lys Ala Gly His Gly Lys Ala Val Asp Trp Trp Ser Leu
              210             215             220
Gly Ile Leu Leu Phe Glu Leu Leu Thr Gly Ala Ser Pro Phe Thr Leu
              225             230             235             240
Glu Gly Glu Arg Asn Thr Gln Ala Glu Val Ser Arg Arg Ile Leu Lys
              245             250             255
Cys Ser Pro Pro Phe Pro Leu Arg Ile Gly Pro Val Ala Gln Asp Leu
              260             265             270
Leu Gln Arg Leu Leu Cys Lys Asp Pro Lys Lys Arg Leu Gly Ala Gly
              275             280             285
Pro Gln Gly Ala Gln Glu Val Lys Ser His Pro Phe Phe Gln Gly Leu
              290             295             300

```

Asp Trp Trp Ala Leu Ala Ala Arg Lys Ile Pro Ala Pro Phe Arg Pro
 305 310 315 320
 Gln Ile Arg Ser Glu Leu Asp Val Gly Asn Phe Ala Glu Glu Phe Thr
 325 330 335
 Arg Leu Glu Pro Val Tyr Ser Pro Ala Gly Ser Pro Pro Pro Gly Asp
 340 345 350
 Pro Arg Ile Phe Gln Gly Tyr Ser Phe Val Ala Pro Ser Ile Leu Phe
 355 360 365
 Asp His Asn Asn Ala Val Met Ala Asp Val Leu Gln Ala Pro Gly Ala
 370 375 380
 Gly Tyr Arg Pro Gly Arg Ala Ala Val Ala Arg Ser Ala Met Met Gln
 385 390 395 400
 Asp Ser Pro Phe Phe Gln Gln Tyr Glu Leu Asp Leu Arg Glu Pro Ala
 405 410 415
 Leu Gly Gln Gly Ser Phe Ser Val Cys Arg Arg Cys Arg Gln Arg Gln
 420 425 430
 Ser Gly Gln Glu Phe Ala Val Lys Ile Leu Ser Arg Arg Leu Glu Glu
 435 440 445
 Asn Thr Gln Arg Glu Val Ala Ala Leu Arg Leu Cys Gln Ser His Pro
 450 455 460
 Asn Val Val Asn Leu His Glu Val Leu His Asp Gln Leu His Thr Tyr
 465 470 475 480
 Leu Val Leu Glu Leu Leu Arg Gly Gly Glu Leu Leu Glu His Ile Arg
 485 490 495
 Lys Lys Arg Leu Phe Ser Glu Ser Glu Ala Ser Gln Ile Leu Arg Ser
 500 505 510
 Leu Val Ser Ala Val Ser Phe Met His Glu Glu Ala Gly Val Val His
 515 520 525
 Arg Asp Leu Lys Pro Glu Asn Ile Leu Tyr Ala Asp Asp Thr Pro Gly
 530 535 540
 Ala Pro Val Lys Ile Ile Asp Phe Gly Phe Ala Arg Leu Arg Pro Gln
 545 550 555 560
 Ser Pro Ala Glu Pro Met Gln Thr Pro Cys Phe Thr Leu Gln Tyr Ala
 565 570 575
 Ala Pro Glu Leu Leu Ala Gln Gln Gly Tyr Asp Glu Ser Cys Asp Leu
 580 585 590
 Trp Ser Leu Gly Val Ile Leu Tyr Met Met Leu Ser Gly Gln Val Pro
 595 600 605
 Phe Gln Gly Ala Ser Gly Gln Gly Gly Gln Ser Gln Ala Ala Glu Ile
 610 615 620
 Met Cys Lys Ile Arg Glu Gly Arg Phe Ser Leu Asp Gly Glu Ala Trp
 625 630 635 640
 Gln Gly Val Ser Glu Glu Ala Lys Glu Leu Val Arg Gly Leu Leu Thr
 645 650 655
 Val Asp Pro Ala Lys Arg Leu Lys Leu Glu Gly Leu Arg Ser Ser Ser
 660 665 670
 Trp Leu Gln Asp Gly Ser Ala Arg Ser Ser Pro Pro Leu Arg Thr Pro
 675 680 685
 Asp Val Leu Glu Ser Ser Gly Pro Ala Val Arg Ser Gly Leu Asn Ala
 690 695 700
 Thr Phe Met Ala Phe Asn Arg Gly Lys Arg Glu Gly Phe Phe Leu Lys
 705 710 715 720
 Ser Val Glu Asn Ala Pro Leu Ala Lys Arg Arg Lys Gln Lys Leu Arg
 725 730 735
 Ser Ala Ala Ala Ser Arg Arg Gly Ser Pro Val Pro Ala Ser Ser Gly
 740 745 750
 Arg Leu Pro Ala Ser Ala Ala Lys Gly Thr Thr Arg Arg Ala Asn Gly
 755 760 765
 Pro Leu Ser Pro Ser
 770

<210> 3
<211> 200
<212> DNA
<213> Artificial Sequence

<220>
<223> Targeting vector

<400> 3
ggctagtttg cactgcaaat tgcttttcag gcttaaccga gcaccctcca gggagggctc 60
accttctcct tagaaggctc tgtcagcctt ttgcaccccc cttcccacca gtctgcaccg 120
ccctcgcaact tacagcccac cttccaaccg cttgcttgct gctcccacag cctatgggaa 180
ggtgttcctg gtgcggaaga 200

<210> 4
<211> 200
<212> DNA
<213> Artificial Sequence

<220>
<223> Targeting vector

<400> 4
cacactgcac tacgccttcc agacggatgc caagctgcac ctcatoctgg gtgagggcct 60
gtcacatctc agctttggag agggacggga gttagattag gctaccttgt agggaaacat 120
ttcaacgtct cctcactccc agactacgtg agcggtggtg agatgttcac tcacctctac 180
cagcgccagt acttcaagga 200

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 March 2003 (06.03.2003)

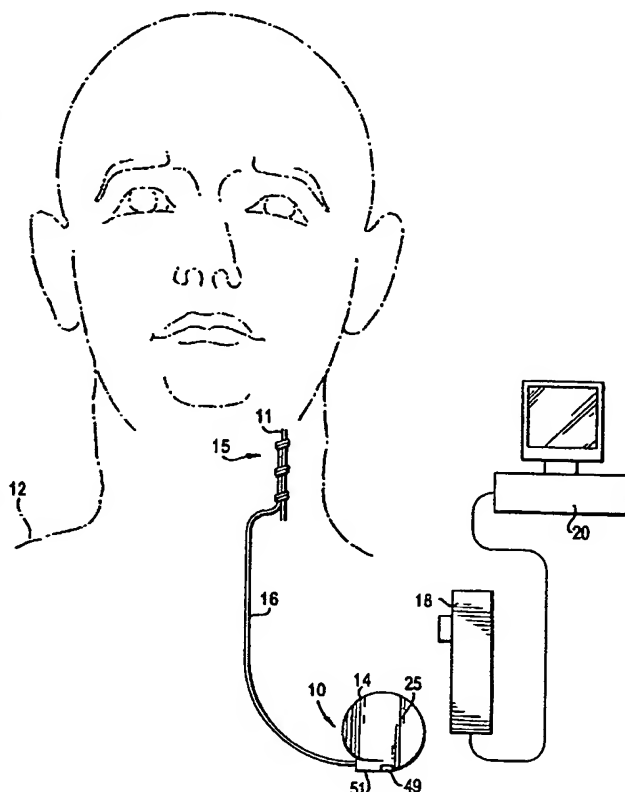
PCT

(10) International Publication Number
WO 03/018108 A3

- (51) International Patent Classification⁷: **A61N 1/36** (74) Agent: **GREENE, Donald, R.**; Blank Rome Comisky & McCauley, Suite 1000, 900 17th Street, N.W., Washington, DC 20006 (US).
- (21) International Application Number: **PCT/US02/25712**
- (22) International Filing Date: **13 August 2002 (13.08.2002)** (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data: **09/933,086** **21 August 2001 (21.08.2001)** **US** (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant: **CYBERONICS, INC.** [US/US]; 16511 Space Center Blvd., Suite 600, Houston, TX 77058 (US).
- (72) Inventors: **TERRY, Reese, S., Jr.**; 2006 Orchard Country Lane, Houston, TX 77062 (US). **ADKINS, Robert, A.**; 5416 North Airline, Angleton, TX 77515 (US). **BARRETT, Burke, T.**; 4403 Prince Pine Trail, Houston, TX 77059 (US).

[Continued on next page]

(54) Title: **TREATMENT OF CONGESTIVE HEART FAILURE AND AUTONOMIC CARDIOVASCULAR DRIVE DISORDERS**



(57) Abstract: A neurostimulator (10) for stimulating the patient's vagus nerve (11) at or above the cardiac branch with an electrical pulse waveform at a stimulating rate sufficient to maintain the patient's heart beat at a rate well below the patient's normal resting heart rate, thereby allowing rest and recovery of the heart muscle, an increase in coronary blood flow, and/or growth of coronary capillaries. A metabolic need sensor detects the patient's current physical state and concomitantly supplies a control signal to the neurostimulator (10) to vary the stimulating rate. If rest is detected, the neurostimulator reduces the patient's heart rate below the patient's normal resting rate. If physical exertion is detected, the neurostimulator increases the patient's heart rate above the normal resting rate.

WO 03/018108 A3



Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(88) Date of publication of the international search report:

31 July 2003

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/25712

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61N 1/36

US CL : 607/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 607/1-2, 4-5, 9, 17, 19, 44, 115, 116, 118; 128/897-898

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,330,507 A (SCHWARTZ) 19 July 1994 (19.07.1994), whole document.	14-18, 20-22, 33
Y		19
X	US 6,141,590 A (RENIRIE et al.) 31 October 2000 (31.10.2000), whole document.	35
X	US 6,073,048 A (KIEVAL et al.) 06 June 2000 (06.06.2000), whole document.	36
Y	US 6,002,963 A (MOUCHAWAR et al.) 14 December 1999 (14.12.1999), whole document.	19

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 December 2002 (10.12.2002)

Date of mailing of the international search report

23 APR 2003

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Kristen L Droesch

Telephone No. 703-605-1185

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)